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The Liberation of Hydrogen Sulphide by X-Radiation from Cysteine and Glutathione

By W. M. DALE and J. V. DAVIES
Christie Hospital and Holt Radium Institute, Manchester

(*Received* 15 *June* 1950)

In continuation of experiments on the effects of ionizing radiations on amino-acids, proteins, peptides, etc. (Dale & Davies, 1949; Dale, Davies & Gilbert, 1949*a*, *b*), we have found that hydrogen sulphide is split off, under certain conditions, from cysteine and glutathione. This may be a result of significance in view of the role attributed to the oxidation of thiol groups in glutathione and proteins for the inactivation of enzymes containing sulphydryl groups (Barron & Dickman, 1949; Barron, Dickman, Muntz & Singer, 1949), and the change of the other cell constituents. It shows in particular that the radiation effect does not consist solely of an oxidation of thiol groups to disulphides, and also shows that in the case of cysteine production of hydrogen sulphide takes place in preference to deamination.

This paper deals with the quantitative relationships obtaining for the liberation of hydrogen sulphide from cysteine and glutathione in aqueous solution, by the action of X-radiation under various conditions.

EXPERIMENTAL

Radiation was obtained from a 500 kV. continuously evacuated X-ray tube (half-value layer 5·2 mm. Cu, average λ=0·056 A.).

The treatment of glassware and the preparations of solutions, etc., followed the lines laid down by Dale, Gray & Meredith (1949).

Irradiation. The solutions were irradiated in sealed, cylindrical gas-washing bottles of soda glass (approximately 2·5 cm. diameter and 10 cm. high) with fused-in tubes. A measured volume of liquid was carefully introduced into the bottle through the inlet tube by means of a narrow-stemmed funnel drawn out from a test tube. If wetting of the inlet tube occurred a spill of filter paper was used to remove any trace of solution from the point where the glass was to be fused. The inlet and outlet tubes were then drawn out to a fine point, sealed, and the bottles irradiated.

Collection and estimation of hydrogen sulphide. The method employed for the determination of H_2S was, in principle, that of Almy (1925) suitably adapted for the present purpose. This method consists essentially of the removal of H_2S by a stream of CO_2, the H_2S being absorbed in zinc acetate solution and estimated colorimetrically as methylene blue. The method proved to be satisfactory, though slight modifications were applied.

After irradiation a scratch was made with a sharp glass knife 0·5 cm. from the sealed ends of each tube. The bottle was then connected to the bubbling train by close-fitting rubber tubing pushed well over the scratch mark, and the glass was broken at the file marks inside the rubber connexions by bending the rubber tubing sharply. The rubber tubing must be of good quality and of wall thickness about 1·5–2 mm. to avoid a puncture during breakage of the glass. It is important that the inlet tube be broken whilst under slight pressure from the CO_2 cylinder before breaking of the outlet tube. In this way the danger of sucking back the irradiated solution was avoided.

In those cases where the pH of the irradiated solution is 5 or greater, it is necessary to introduce acid into the solution in order to drive over the H_2S present. This was done by placing a T-piece in the train between the CO_2 cylinder and the gas-washing bottle. The side arm of the T-piece was connected to a small piece of narrow-bore (3 mm.) rubber tubing fitted with a screw clip. The nozzle of a calibrated all-glass syringe was inserted into the rubber tube, the clip opened, and a measured volume of HCl forced into the system while the stream of gas was already running. The injected fluid was thus swept into the washing bottle by the current of CO_2. The volume and strength of acid injected was adjusted so as to bring the mixture in the bottle to approximately pH 2. When this procedure was applied to the unirradiated solutions zero blanks were obtained.

During the preparation of the calibration curve it was found that the colour depth produced on direct addition of the reagents to a standard sulphide solution always slightly exceeded that obtained on driving over the H_2S by CO_2; i.e. there appeared to be some slight loss in the transfer process. However, this difference was only small and always strictly reproducible, so that no error was introduced in reading from a calibration curve constructed under the exact experimental conditions of transfer by bubbling.

We found that the standard H_2S solutions as recommended by Almy were not entirely satisfactory and, after some trials, adopted the following procedure; 15 ml. of a 20 % (w/v) zinc acetate solution was made up to approximately 430 ml. in a 500 ml. volumetric flask and sufficient aqueous H_2S added to bring the final concentration of ZnS to roughly 6 μg./ml. when made up to 500 ml. This solution was then accurately titrated by the iodate-iodide method. The concentration of ZnS must not greatly exceed 5–6 μg./ml. or slow precipitation will occur.

The amount of fluid irradiated in the bottle was 10 or 25 ml. depending on the yield of H_2S expected. The H_2S was trapped in two filter tubes in series, the first trap containing 10 ml. and the second 5 ml. of zinc acetate solution. At the con-

clusion of bubbling the trapping fluid was quantitatively transferred with 2 or 3 ml. of water into a 25 ml. flask, the reagents added, and the colour allowed to develop for the prescribed time before the volume was made up to the mark and read.

The zinc acetate absorbing solution was of 2% concentration, made by diluting a 20% solution ten times and adding 0·1 ml. N-acetic acid to every 100 ml.; the addition of the acetic acid clears any slight opalescence due to $Zn(OH)_2$.

Colorimetry. The methylene blue solutions were measured in a Hilger 'Spekker' absorptiometer using no. 608 red filters, and 4 cm. cells. We found that the most suitable colour intensities giving the greatest sensitivity for reading were those corresponding to H_2S concentrations in the range 0·05–0·20 μg./ml. After a little experience it can be seen at a glance whether the colour produced is in this range; if the colour was too intense it was suitably diluted with water containing the reagents in the requisite proportions.

RESULTS

Cysteine hydrochloride

The production of H_2S for any one concentration of cysteine hydrochloride is directly proportional to the dose up to 100,000 r. (Fig. 1) which was the highest dose applied.

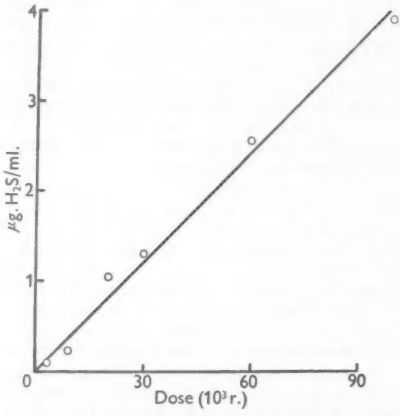

Fig. 1. The yield of H_2S in the X-radiation of cysteine as function of the radiation dose. Cysteine hydrochloride (concentration 7500 μg./ml., pH 2).

Effect of oxygen and of hydrogen peroxide. Solutions saturated with air yield the same amount of H_2S as those which have been highly evacuated; the process therefore seems to be independent of the oxygen content of the solution. Solutions of cysteine hydrochloride were made containing hydrogen peroxide in such concentrations (0·001 M) as would be produced by the X-ray dose used (Bonet-Maury & Lefort, 1948). With these solutions less than 2% of the H_2S yield was obtained in an equivalent time and even ten times the expected hydrogen peroxide concentration produced only one-fifth the yield of H_2S produced by irradiation.

Effect of concentration of cysteine hydrochloride. The yield of H_2S for a given X-ray dose rises with increasing concentration of cysteine hydrochloride solution in a way that is similar to, but not identical with, the production of ammonia from glycine and serine, a flat maximum yield being obtained in the concentration region 1–20% (Fig. 2). Cysteine

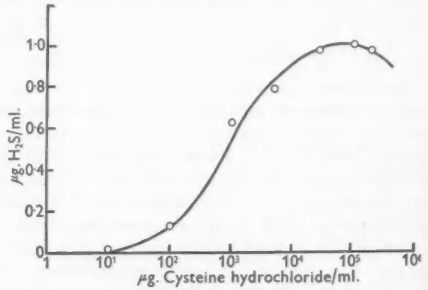

Fig. 2. The yield of H_2S in the X-radiation of cysteine as function of the concentration of cysteine hydrochloride (dose 33,000 r., pH 2).

hydrochloride is extremely soluble, and solutions of very high concentrations (maximum 51·8% at 18°) showed a pronounced falling off in the H_2S yield. The curve shows the total yield from direct as well as indirect effect of radiation. The efficiency of H_2S production is, in general, less than that of ammonia formation from amino-acids. The maximum ionic yield obtained in the concentration region 1–20% is approximately 0·3; the corresponding yield of ammonia from glycine varies from 1 to 3 according to the concentration of glycine (Dale *et al.* 1949*a*).

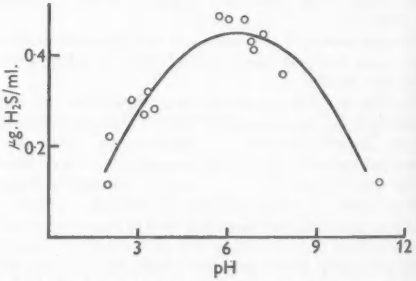

Fig. 3. The yield of H_2S in the X-radiation of cysteine as function of pH. Cysteine hydrochloride (concentration 5000 μg./ml., dose 9500 r.).

Effect of pH. The production of H_2S from cysteine by X-radiation is dependent on pH (Fig. 3) with a maximum at about pH 6. Cysteine, of course, is rapidly oxidized to cystine in alkaline solution, and

in order to study H_2S liberation from cysteine at a pH > 7, sodium pyrophosphate was added as an inhibitor of autoxidation (Warburg & Sakuma, 1923). Control experiments showed that sodium pyrophosphate in M/25 and M/60 concentration had no effect in the production of H_2S by radiation.

In an earlier communication (Dale *et al.* 1949*a*) on ammonia production by X-rays from a wide variety of amino-acids, etc., an effect of pH was noted and this was confirmed by Stein & Weiss (1949). In particular, it was observed that the ammonia yield from cysteine hydrochloride at pH 2 was nil or very small with doses of the order of 10^5 r. It was therefore important to know whether deamination follows the same course as liberation of H_2S over an extended range of pH. It was found that at pH 2, 4 and 6 no ammonia is produced whereas the H_2S production rises to a maximum at about pH 6·5.

Cystine

Cystine can only be studied in either strongly acid or alkaline solution because of its extreme insolubility at neutral pH. In acid solution no liberation of H_2S was observed with the doses used. There was, however, some evidence that in alkaline solution H_2S was produced in very small yield, although the accuracy of the result was lessened by the presence of a large blank; this blank could not be eliminated by recrystallization, and was small when estimated immediately after the solution was made up. It appears, therefore, to be due chiefly to the chemical action of the alkali. It is a well known fact, of course, that cystine loses sulphur, so-called 'labile sulphur', on treatment with alkali, even mild alkali such as sodium carbonate; see, for example, Andrews (1930) and Sheppard & Hudson (1930).

Commercial cysteine hydrochloride was used in the majority of these experiments, and some slight variation in H_2S yield was noted in different batches. Accordingly, attempts were made to purify the acid by recrystallization from concentrated hydrochloric acid (du Vigneaud, Audrieth & Loring, 1930). The crystalline form and appearance of the product appeared to be improved, but melting point and H_2S yield were not materially altered. It should be mentioned that the commercial product gave a zero blank value.

Glutathione

We have found that this biologically important tripeptide also yields H_2S on X-radiation, as might be expected on account of its relation to cysteine. For a solution containing 9500 μg./ml. of glutathione the ionic yield is about 0·25, almost the same as that for an equimolar solution of cysteine hydrochloride (5000 μg./ml.) at pH 2. Fig. 4 shows for reduced glutathione the relationship between pH and H_2S liberation by X-rays. In general, the yield of H_2S rises in the range pH 2–6·5, although an unexpected depression in yield is observed at about pH 5·5.

The oxidized form of glutathione in the same molar concentrations and treated with similar X-ray doses showed no liberation of H_2S, again showing analogous behaviour to the cysteine-cystine system.

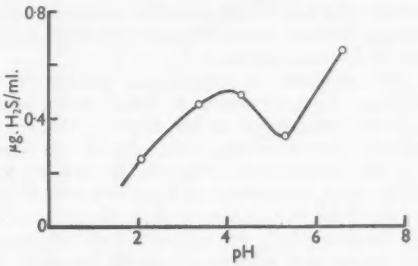

Fig. 4. The yield of H_2S in the X-radiation of reduced glutathione as function of pH. Reduced glutathione (concentration 9500 μg./ml., dose 12,800 r.).

DISCUSSION

It is becoming more and more apparent that sulphur in organic linkage, as well as in its elemental form and in the form of thiosulphate (Dale, 1947; Dale, Davies & Meredith, 1949; Barron & Dickman, 1949; Barron *et al.* 1949; Mole, Philpot & Hodges, 1950; Patt, Tyree, Straube & Smith, 1949; Chapman, Sipe, Eltzholtz, Cronkite & Chambers, 1949) plays an important part in radiation chemistry and radiation biology. Barron and his co-workers consider the oxidation of the thiol form of glutathione and proteins to the disulphide form (a reaction which can be reversed chemically) to be the biologically important step. Others have not investigated this first oxidation step, but rather the destruction of glutathione by X-rays, γ-rays, etc. (Hammett, 1932; Woodward, 1933; Kinsey, 1935), i.e. an irreversible change which may be an oxidation to a further stage or a complete unspecified breaking-up of the molecule. Our experiments have shown that, in addition to any reversible oxidation which may occur, there are irreversible changes leading to the liberation of hydrogen sulphide. We do not know as yet whether this is due to an oxidation or reduction, but experiments are in progress to attempt to solve this problem.

Because of the technique used in previous studies by Kinsey (1935), Hammett (1932) and Woodward (1933) the H_2S reaction escaped detection. These investigators were compelled to use low concentrations in order to be able to measure an at least 10% change in the original concentration with any degree of accuracy. They, therefore, only registered an overall change in concentration of thiol-reacting

material. If, however, reaction product is measured, as in the present investigation, a minute fraction of 1 % of the original thiol groups can be determined with accuracy.

It is further of interest that although all the other amino-acids investigated (Dale *et al.* 1949*a*) show deamination by X-radiation in varying degree, cysteine was not deaminated. The radiation energy seems to be used, partly at least, for H_2S production and not for deamination.

The instability of glutathione, particularly its property of readily losing its sulphydryl group as H_2S was commented on by Hopkins (1929). He showed, for example, that during prolonged aeration of an aqueous solution of the reduced form at pH 7·6 (in the presence of a trace of iron) 80 % of the thiol form was converted to the disulphide form. The remaining 20 % was proved to have lost some of its nitrogen and sulphur. It should be noted that under the conditions of our work no H_2S was detected in the control experiments.

The majority of experiments was deliberately done with cysteine because of its greater stability and the easy suppression of autoxidation in this case. However, the experiments with glutathione prove the essentially similar response to radiation with the one peculiar exception of a depression in the pH curve near pH 5·5. One has to bear in mind, however, that at pH values near neutrality the stability of glutathione is greatly diminished.

It seems that the liberation of H_2S may be of importance from the biological point of view since its occurrence within the cell is likely to have a toxic effect.

SUMMARY

1. Experiments are described on the production of hydrogen sulphide by X-radiation from cysteine and glutathione.

2. The influence of pH, oxygen, hydrogen peroxide, concentration of solute, etc., on the process, has been studied.

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An L-Glutamic Acid Decarboxylase from Barley

By H. BEEVERS
Oxford Medicinal Plants Scheme, Department of Botany, University of Oxford

(*Received* 29 *June* 1950)

Okunuki (1937) discovered an enzyme in powders prepared from various plant tissues which was specific for the decarboxylation of glutamic acid. He reported that pollen from *Lilium auratum* was a particularly good source of the enzyme, whereas barley seedlings were included in a list of tissues which did not yield active preparations by his method. Schales, Mims & Schales (1946) have since shown that the enzyme can be extracted by water fairly readily from plant tissues and give a list of some thirty species from which it was obtained. Using aqueous extracts from carrots, Schales & Schales (1946*a*) have investigated the characteristics of the enzyme and the reaction kinetics. The present paper describes some experiments on a similar enzyme occurring in seedling barley roots.

EXPERIMENTAL

Preparation of enzyme. Spratt Archer barley seed, previously soaked for 24 hr., was germinated in thick layers in rectangular glass vessels with the minimum amount of water. In 3 days at room temperature the growing roots had bound the grains together in mats, which were then supported on bent glass stands 2·5 cm. high. The seedlings were maintained in an incubator at 21–24° and were well watered. In this way a heavy crop of roots was obtained which grew rapidly downwards away from the grain. The seedlings continued to grow well for about 15 days, but in most cases the roots were removed after 10 days. They were cut off with a razor, washed in several changes of distilled water and then either (*a*) treated in a Waring blender with water or a similar amount of 0·5 M-phosphate buffer, or (*b*) ground with a minimum amount of water or buffer in a pestle and mortar. The fibrous residue was separated on muslin and discarded and the remaining solid material centrifuged down at 3000 rev./min. The cloudy supernatant was collected and stored at 0° until required. More concentrated extracts were occasionally prepared by method (*a*) by blending further roots in the liquid obtained in the first extraction.

Manometry. Conventional Warburg technique was employed to measure CO_2 outputs at 30° after addition of glutamate from the side arms at zero time. The solution containing the enzyme was placed in the main chamber of the vessel and was maintained at pH 5·2 by addition of 0·5 M-phosphate buffer. The gas phase in the experiments reported here was air, since some preliminary comparisons had shown that there was little difference in activity under aerobic and anaerobic conditions.

RESULTS

Experiments with untreated aqueous extracts

Effect of amount of enzyme on decarboxylation. The extracts alone showed a very slow evolution of CO_2 and the addition of glutamate immediately led to substantial increases in the rate, whereas additions to boiled extracts were without effect. Fig. 1 shows the result of an experiment in which sufficient glutamate to give a final concentration of 0·01 M was added to different amounts of extract. In all cases the decarboxylation slowed down well before the theoretical yield of CO_2 (560 μl.) had been evolved; there was only 56 % decarboxylation in *a* which received 2 ml. of extract, four times the amount supplied in *c*. Okunuki (1937) and Schales *et al.* (1946) observed that curves of this type were typically produced in their experiments with aqueous extracts and that the CO_2 yields were frequently less than one-third of those equivalent to the added glutamate. In considering their suggestions for the cause of this, it is significant that, with increasing amounts of enzyme, the decarboxylation proceeded further. More concentrated extracts have since been obtained in which the CO_2 output in 60 min. was 80 % of that calculated (Fig. 2).

Stability of the enzyme during the reaction. Samples from different batches of roots varied in their ability to withstand a prolonged period at 30°, even in the absence of substrate. It was sometimes found that, if the addition of the glutamate was delayed for 2–3 hr., considerable thermal inactivation had

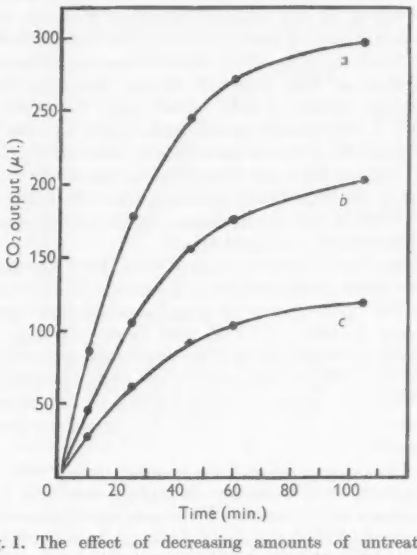

Fig. 1. The effect of decreasing amounts of untreated extract on the decarboxylation of 0·01 M-glutamate: *a*, 2 ml. extract; *b*, 1 ml. extract; *c*, 0·5 ml. extract. Volumes adjusted with 0·5 M-phosphate, pH 5·2.

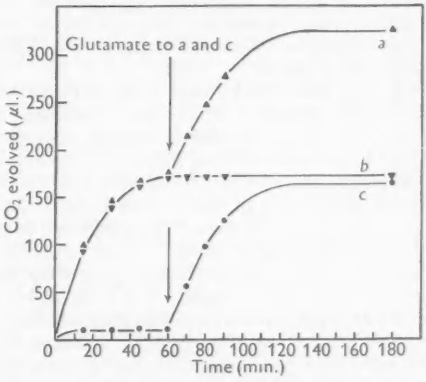

Fig. 2. The effect of delayed addition of glutamate to 2 ml. extract: *a* and *b* received 0·5 ml. 0·02 M-glutamate at the outset, *c* had no addition; 0·5 ml. 0·02 M-glutamate was added to *a* and *c* after 60 min., but not to *b*.

occurred, whilst in other cases only small losses were encountered during the same period. The results of an experiment designed to test the possibility that the enzyme was inactivated during the reaction or inhibited by accumulation of end product are shown

in Fig. 2. To two samples, *a* and *b*, of a concentrated extract was added 0·5 ml. of 0·02 M-glutamate, while to a third, *c*, there was no addition at the outset. There was a vigorous CO_2 output from *a* and *b* and at the end of 60 min., when this was slowing down, a further 0·5 ml. of glutamate was added to *a* and also to *c*. The CO_2 output from *c* showed that some thermal inactivation had occurred during the first hour (30 min. output 115 μl., initial value for *a* and *b* 143 μl.). The similar output from *a* after the second addition (99 μl. in the period 60–90 min.) shows that the enzyme had not been appreciably inactivated during the decarboxylation and was not markedly inhibited by the accumulation of end product which had occurred in the first 60 min.

The effect on activity of age of roots. Extracts made by method *a* from roots of different ages did not vary greatly. The activity of samples taken daily from a crop between the fifth and thirteenth days of growth, expressed as μl. CO_2 from 0·002 M-glutamate/mg. dry weight of root/10 min., ranged between 2·0 and 3·2. Second extracts made from the residues showed about one-third of the activity of the initial extracts.

Attempts to precipitate the enzyme. Gale (1946) has employed the technique of drying bacterial suspensions with acetone as the principal method of preserving the decarboxylases in a solid state. When water extracts containing enzyme from barley were treated with acetone, a precipitate formed which was quite inactive towards glutamate when dried and redissolved in buffer. Similarly, dry powders prepared from roots by acetone treatment did not contain active enzyme. Ethanol treatment also yielded inactive preparations.

The only precipitant found to be effective was ammonium sulphate. Precipitates obtained by adding an excess of ammonium sulphate to extracts showed considerable activity when redissolved, even without prior removal of salt included with the unwashed precipitate. In one such experiment 62% of the total activity of the extract was found in the ammonium sulphate precipitate. Since no increase in activity occurred when the solutions of the precipitates were dialysed against water for 24 hr., it seems that small amounts of ammonium sulphate do not affect the activity of the enzyme. It has, in fact, been shown that ammonium sulphate in concentrations as high as 0·2 M has little inhibitory effect; in one experiment the CO_2 output from 0·004 M-glutamate in 30 min. was reduced from 117 to 103 μl. Prolonged contact with the saturated salt solution is not injurious, and the best means of preserving the enzyme found so far has been to leave it as a precipitate in the ammonium sulphate solution at 0° until required. It is stable for at least 14 days in this condition. Little separation from other proteins was achieved by using different ammonium sulphate concentrations, as almost all of the proteins, including the decarboxylase, precipitated at 25–50% saturation. Attempts to separate the enzyme by precipitation at different pH values were made with little success. Some precipitates formed at pH 3·2 and 8·5, but they did not contain the enzyme, and experiments on the supernatant re-adjusted to pH 5·0 showed that losses in activity of over 70% had occurred at these pH levels. Most of the further work was carried out using the crude precipitate, prepared by saturating the water extract with ammonium sulphate, dissolved in 0·5 M-phosphate buffer pH 5·2. Such preparations had the following advantages over aqueous extracts: (1) Samples of closely similar activity could be taken from a bulk preparation on successive days. (2) Considerable concentration of the enzyme could be achieved. (3) Water-soluble amino-acids, known to be present in the original extracts, did not occur in the precipitate.

Experiments using redissolved precipitated enzyme

Course of reaction. When glutamate was added, the initial part of the CO_2 output curve was linear and, in contrast to the behaviour of untreated extracts, did not show early flattening. In Fig. 3 are shown CO_2 outputs with three different (laboratory reagent) preparations of glutamate, and it is seen that the decarboxylation was maintained at a falling rate until 88–94% of the calculated yield of CO_2 had been produced in 240 min.

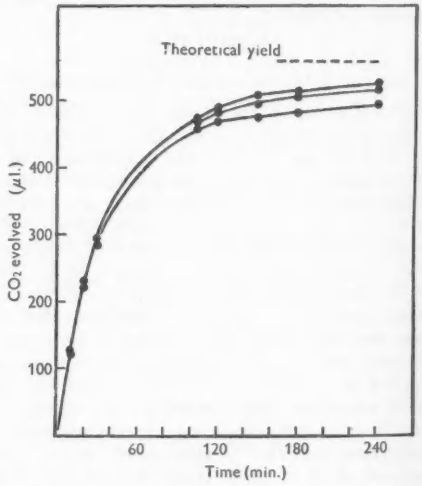

Fig. 3. The course of decarboxylation by 2 ml. redissolved $(NH_4)_2SO_4$ precipitate of three different preparations of glutamate. Concentration of substrates 0·01 M.

Effect of pH. Samples (1 ml.) of a precipitate which had been redissolved in water were added to 1 ml. of 0·5 M-buffer at various pH values in manometers. CO_2 outputs during the first 10 min. after tipping 0·5 ml. of 0·1 M-glutamate were recorded and the amounts by which these exceeded outputs from control systems at the same pH have been used to establish the pH activity curve shown in Fig. 4. No correction has been made for CO_2 retained at the higher pH levels. The optimum pH range is 5·0–5·5, which is near the values previously reported, and there is little activity outside the range pH 3·5–7·5.

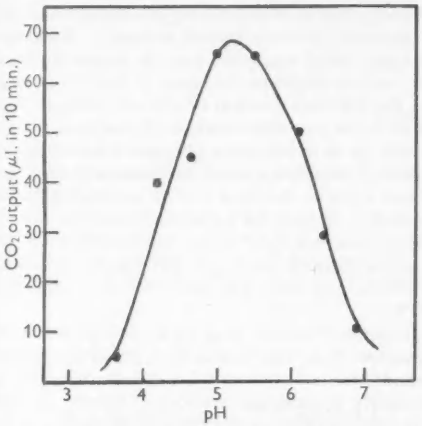

Fig. 4. The effect of pH on the activity of the enzyme. The points are 10 min. CO_2 outputs from 0·02 M-glutamate.

Effect of substrate concentration. This section has been written in conjunction with W. L. Howard, Rhodes Scholar from the University of Oklahoma, U.S.A., at present in the Department of Botany, Oxford. Schales and his colleagues have generally used concentrations of glutamate of about 0·06 M in their experiments and have reported (Schales & Schales, 1946*a*) that the enzyme from carrots has a Michaelis constant of $3·6 \pm 0·4 \times 0·001$ M. In making their calculations these authors have introduced methods to correct for the fact that their preparations gave rates of CO_2 output which were already falling during the first few minutes of the reaction.

With the previously precipitated enzyme the CO_2 outputs were virtually linear at the outset and 10 min. values provide a valid measure of the initial rate. A series of experiments with graded glutamate concentrations gave the results shown in Fig. 5. The points are the observed initial rates at the corresponding glutamate levels and it is clear that they increase to a maximum and then decrease as the substrate concentration increases, indicating inhibition by excess substrate. The activity-substrate concentration curve is clearly not a rectangular hyperbola, and calculations based on the application of the simple Michaelis formula are not possible in the case of the barley enzyme.

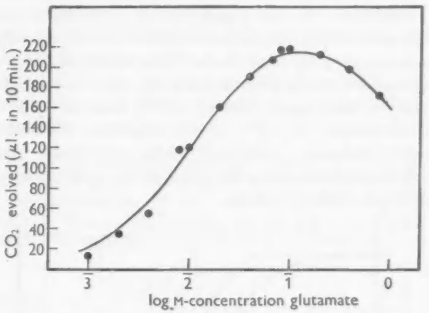

Fig. 5. The effect of glutamate concentration on the rates of decarboxylation. The points are the observed 10 min. CO_2 outputs. The line is calculated from the equation

$$v = V[S]/([S] + K_s + [S]^n/K_2),$$

using the values given in the text.

Using the analysis proposed by Lineweaver & Burk (1934), the data can be shown to agree with the mechanism of inhibition by formation of a complex of one enzyme molecule with two of substrate,

$$E + S \rightleftharpoons ES \rightarrow P; \quad ES + S \rightleftharpoons ES_2 \text{ (inactive)},$$

the kinetics of which is described by the equation

$$v = V[S]/([S] + K_s + [S]^n/K_2),$$

where v = initial velocity, V = maximum initial velocity, K_s = dissociation constant of the enzyme-substrate complex (ES), n = number of substrate molecules in the inactive complex and K_2 = dissociation constant of this inactive complex. The curve in Fig. 5 is calculated from this equation using the values $V = 245$ μl./10 min., $K_s = 9·6 \times 0·001$ M, $K_2 = 1·93$ M and $n = 2$ which have been determined from the primary data. The close agreement of the observed points and the calculated curve strongly indicates the correctness of the proposed mechanism and the reliability of the calculated constants.

Effect of temperature on stability. When redissolved, the enzyme lost activity over a period of days, even when stored at 0°, and was very rapidly inactivated at temperatures above 50°. Fig. 6 shows the effect of pretreatment for (*a*) 5 min. and (*b*) 10 min. at various temperatures on the activity of the enzyme when it was subsequently measured at 30°. It is clear that some destruction results at temperatures well below 50° during relatively short exposures. Attention has already been drawn to the losses which occur in experiments at 30°. At 37°, therefore, the temperature used by the American authors, even greater losses might be expected.

Specificity. The enzyme did not decarboxylate the closely related compounds glutaric acid and glutamine, and also had no measurable effect on the following amino-acids: L-arginine, L-aspartic acid, L-histidine, L-tryptophan and L-tryosine.

Inhibitors. In its reactions to inhibitors the behaviour of the enzyme was closely similar to the examples already described. Thus, at pH 5·2 in the presence of 0·002 M-semicarbazide the rate of decarboxylation was reduced by 86 % and, in 0·001 M-semicarbazide, to 63 % of the control. 0·00012 M-Hydroxylamine produced 94 % inhibition and 0·0004 M-cyanide complete inhibition. Carbon monoxide was without effect.

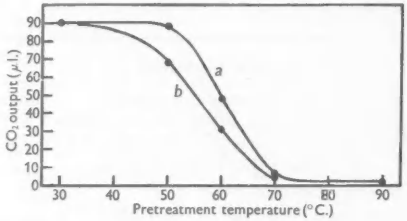

Fig. 6. The effect of pretreatment at the temperatures indicated for (*a*) 5 min. and (*b*) 10 min. on the subsequent activity of the enzyme measured at 30°. The points represent 10 min. CO_2 outputs from 0·02 M-glutamate.

Identity of the product of decarboxylation. Gale (1940) has shown that γ-aminobutyric acid is the product when glutamic acid is attacked by the bacterial carboxylase, and this acid has now been satisfactorily identified from barley enzyme digests.

On paper chromatograms with phenol-water as the mobile phase, it was found that crude water extracts from barley contained several amino-acids, or ninhydrin-reacting substances, which tended to mask on the paper the effects of added glutamate. The previously precipitated enzyme, however, did not contain any free amino-acids and gave clear blanks on the chromatograms. Samples were removed at intervals from a flask in which decarboxylation by this enzyme preparation was proceeding at a known rate, and run on chromatograms. It was found that as the reaction progressed a ninhydrin-reacting substance appeared at R_F 0·72 and increased in intensity while the glutamate spot (R_F 0·27) was disappearing. When decarboxylation was complete, the sample showed that the glutamate had entirely disappeared and that the spot at R_F 0·72 was then strongly developed and was the only one present. Parallel runs with γ-aminobutyric acid showed identical R_F values and only one spot was observed on the chromatogram when this acid was added to a digest sample which had completely decarboxylated the added glutamate.

DISCUSSION

The demonstration of an active glutamic acid decarboxylase in barley, after Okunuki's negative finding, can probably be explained by the fact that different methods of extraction were employed. Schales *et al.* (1946) have already shown that Okunuki failed to detect the enzyme in some tissues because he used preparations from which soluble matter had been removed, and the barley enzyme is quite soluble.

The enzyme is fairly stable for short periods at 30°, but thermal inactivation may be one of the contributory factors when incomplete decarboxylation is observed with untreated extracts. Schales & Schales (1946*a*) suggested that the accumulation of end product might be the cause of the falling rates, but the inhibitory action of the end product (now shown to be γ-aminobutyric acid) has been demonstrated to be small, since glutamate added after a period of decarboxylation was attacked at a rate almost equal to that in a control system. A further possibility is that, in extracts possessing low decarboxylase activity, other reactions which also consume glutamic acid may significantly reduce the substrate concentration and therefore the yield of CO_2.

It seems, however, that if sufficiently active preparations (e.g. the redissolved precipitates mentioned) are used, quantitative decarboxylation can be nearly approached without difficulty. Schales & Schales (1946*b*) have now shown that complete and specific decarboxylation of glutamate can be achieved using preparations obtained by freeze drying squash tissue.

In calculating a value for the Michaelis constant, using experimental data for the redissolved enzyme, it was found necessary to make allowance for inhibition by excess substrate. A value somewhat higher than that recorded for the carrot enzyme was obtained. A consequence of the relatively high Michaelis constant is that the rate of CO_2 output begins to fall some time before decarboxylation nears completion. The evidence of the different Michaelis constants alone does not show that the carrot and barley enzymes are basically different, although this remains a possibility. In many of their other properties the enzymes are quite similar.

The wide occurrence of glutamic acid decarboxylase (Schales *et al.* 1946) and its substrate in plants in all probability explains the origin of γ-aminobutyric acid. This acid has now been isolated in quantity from beetroot (Westall, 1950) and also from apples (Hulme & Arthington, 1950). Dent (1948) gives evidence for supposing that an unidentified component of the amino-acids from potato (described earlier by Dent, Stepka & Steward, 1947) is γ-aminobutyric acid. It might be expected that the more

precise methods of analysis now available will reveal the widespread occurrence of γ-aminobutyric acid in small amounts.

SUMMARY

1. Aqueous extracts from barley roots contain an active L-glutamic acid decarboxylase, but do not decarboxylate L-aspartic acid, L-arginine, L-histidine, L-tryptophan or L-tryosine at measurable rates.

2. Active precipitates can be obtained from aqueous extracts by treatment with ammonium sulphate, and the enzyme is stable in this state.

3. Almost quantitative decarboxylation can be achieved using redissolved precipitates and the product has been identified by chromatography as γ-aminobutyric acid.

4. A calculation of the Michaelis constant has given a value of $9{\cdot}6 \times 0{\cdot}001$ M, allowance being made for inhibition by high substrate concentrations.

5. The pH relationships and reactions to inhibitors of the barley enzyme are similar to those previously described.

6. It is suggested that the natural occurrence of γ-aminobutyric acid may be ascribed to the functioning of the enzyme *in vivo*.

The author wishes to express his thanks to Dr W. O. James under whose general direction the work was carried out. He is also indebted to Mr R. G. Westall, Low Temperature Research Station, Cambridge, for the gift of a sample of γ-aminobutyric acid.

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The Component Acids and Glycerides of a Horse Mesenteric Fat

By S. S. GUPTA and T. P. HILDITCH
Department of Industrial Chemistry, University of Liverpool

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Until recently no comprehensive account of the component acids of fats from the horse has been recorded. Nevertheless, the general studies of Heiduschka & Steinruck (1921) and of Schuette, Garvin & Schwoegler (1934) showed that horse body fats differed strikingly from those of oxen or sheep in containing relatively small proportions of stearic acid and definite amounts of the linoleic (octadeca-9-12-dienoic) and linolenic (octadeca-9:12:15-trienoic) acids which are present in grass fats (and indeed in many other vegetable fats):

	% (w/w)				
	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Horse body fat (Heiduschka & Steinruck, 1921)	29	7	55	7	2
Horse abdominal fat (Schuette *et al.* 1934)	28	5	50	13	4

A detailed analysis of horse milk fat (Hilditch & Jasperson, 1944) showed its acids to contain 16% palmitic, 3% stearic, 19% oleic, 8% linoleic and 16% linolenic acids (in addition to 22% of saturated acids of lower molecular weight than C_{16} acids, and 11% of unsaturated acids of lower molecular weight than C_{18} acids): the low proportion of stearic acid, and the presence of linoleic and linolenic acids, are again noteworthy and contrast with the higher proportions of the former acid, and the substantial absence of linoleic and linolenic acids, in cow or sheep milk fats.

It was thus felt desirable to undertake a further detailed examination of a horse body fat, not only in relation to its component fatty acids, but especially in regard to its component glycerides. Whilst this work was in progress two further important studies of horse fat component acids have appeared, in both of which the more recent techniques of separa-

tion of the fatty acids by low-temperature crystallization, ester fractionation, and spectrophotometric analysis of mixed oleic, linoleic and linolenic acids have been utilized. Holmberg & Rosenqvist (1949) found that Swedish horse kidney and bone fats contained fairly large proportions of linoleic and 2–4 % of linolenic acids, with significant proportions of myristic (tetradecanoic), palmitoleic (hexadec-9-enoic) and unsaturated C_{20} acids. Brooker & Shorland (1950), in the case of offal, bone and hoof fats from New Zealand horses, also observed the presence of myristic, palmitoleic and unsaturated C_{20} acids, but recorded only about 5 % of linoleic acid and about 16 % of linolenic acid. Both groups of workers reported oleic (34–42 %) and palmitic (18–28 %) as the main component acids of the fats. Their data are discussed more fully later in this paper (Table 6).

The present study was based on a specimen of adipose tissue from the mesentery of a normal healthy horse, which was obtained for us by the Department of Veterinary Science of the University of Liverpool. The mesenteric fat, extracted from the tissues by acetone, was a clear yellow material, semi-liquid at the ordinary temperature; it had an iodine value of 95·6, saponification equivalent 283·1, and contained 0·2 % of free fatty acid (calculated as oleic acid).

METHODS

Component fatty acids. The mixed fatty acids from the oil were first divided into four groups of differing mean unsaturation (*A*, *B*, *C* and *D*) by crystallization from acetone and then from ether at low temperatures. Each group of acids finally obtained was separately converted into methyl esters, which were fractionally distilled in a vacuum through an electrically heated and packed column. The composition of the mixtures of mono-, di- and tri-ethylenic acids present was determined, after alkali-isomerization under standard conditions (Hilditch, Morton & Riley, 1945) from the extinction coefficients of the ultraviolet absorption bands developed at 268 and 234 mμ., and from the I_2 value of the mixture under examination. When only oleic, linoleic and linolenic acids are present, this is most conveniently done by spectrophotometric analysis of each group of acids as a whole; in the present instance unsaturated acids of the C_{16} series were present in addition to the unsaturated C_{18} acids, and it was therefore necessary to carry out the spectrophotometric analyses on the acids from selected ester fractions in which the unsaturated components were seen to belong wholly to either the C_{18} or the C_{16} series of acids. The presence of natural ('seed fat') linoleic and linolenic acids was confirmed by the isolation of their respective characteristic crystalline tetrabromo- and hexabromo-adducts.

Group	Description
A	Insoluble in ether at −30°
B	Soluble in ether at −30°
C	Insoluble in acetone at −70°
D	Soluble in acetone at −70°

The requisite data were then available for calculation of the composition of each ester fraction, therefrom the composition of each group of separated acids, and thence, finally, the component acids of the original horse mesenteric fat.

Component glycerides. The mesenteric fat was crystallized systematically from solutions in acetone or in ether at a range of temperatures from −65 to 0° until little further separation could be observed (by I_2 value). The original fat of I_2 value 95·6 was thus resolved into nine groups of glycerides (A–I), the I_2 values of which ranged from 13·8 to 184·0.

Seven of the nine groups of glycerides were hydrolysed and their mixed fatty acids separately analysed by methods similar to those outlined above; the preliminary resolution by solvent separation was, however, limited to crystallization of the acids from ether or acetone at about −40°, and omitted altogether in the case of the glyceride groups A, B, F and G (Fig. 2, Table 4). The amounts of the two most unsaturated groups were too small for complete analysis by these methods, and their examination was confined to spectrophotometric analysis.

Each individual group of glycerides being relatively simple in composition, it becomes possible to deduce from the component fatty acids present the general composition of the mixed glycerides in each group, and therefrom in the whole fat.

RESULTS

Component fatty acids of horse mesenteric fat

The mixed fatty acids (136·5 g.) obtained by hydrolysis of about 145 g. of the horse fat were systematically crystallized from 10 % solutions in acetone or ether at low temperatures according to the scheme in Fig. 1. Four groups of acids were finally obtained:

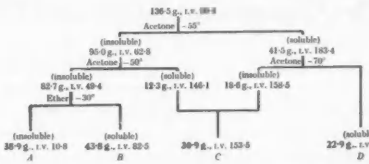

Fig. 1. Crystallization of mixed fatty acids of horse mesenteric fat. I.V. means iodine value.

	Weight		Iodine
	(g.)	(% of total)	value
A	38·9	28·5	10·8
B	43·8	32·1	82·5
C	30·9	22·6	153·5
D	22·9	16·8	205·6

The ester-fractionation data for the methyl esters of each of the four groups of acids are summarized in Table 1.

The acids from selected ester fractions from groups *B*, *C* and *D*, in each of which the unsaturation was clearly due to acids of the C_{18} series, and one from group *D* in which the unsaturated acids were wholly of the C_{16} series, were analysed spectrophoto-

metrically after isomerization with alkali under the standard conditions described by Hilditch *et al.* (1945), with the results summarized in Table 2.

Table 1. *Fractionation of methyl esters of horse mesenteric fatty acids A, B, C, D*

Fraction	Weight (g.)	Saponification equivalent	I_2 value
	Methyl esters of acids *A*		
*A*1	1·03	247·0	0·9
*A*2	2·62	265·0	1·3
*A*3	3·57	269·4	1·4
*A*4	4·10	269·5	1·1
*A*5	4·40	270·3	1·7
*A*6	3·00	271·4	1·7
*A*7	4·19	287·3	31·1
*A*8	3·16	303·9*	33·4
Total	26·07		
	Methyl esters of acids *B*		
*B*1	1·49	245·1	5·8
*B*2	1·95	270·4	32·1
*B*3	3·18	280·9	61·1
*B*4	3·80	294·4	84·7
*B*5	3·63	294·1	90·4
*B*6	5·86	296·2	91·0
*B*7	4·33	296·2	90·4
*B*8	2·04	296·1	86·8
*B*9	1·70	310·5*	84·3
Total	27·98		
	Methyl esters of acids *C*		
*C*1	0·97	240·0	38·2
*C*2	2·98	262·0	89·4
*C*3	2·97	276·5	133·2
*C*4	2·79	286·1	160·5
*C*5	3·25	294·2	162·8
*C*6	3·25	294·3	167·5
*C*7	2·29	294·6	165·8
*C*8	2·31	294·1	168·8
*C*9	2·39	294·9	167·3
*C*10	2·30	326·8*	146·3
Total	25·50		
	Methyl esters of acids *D*		
*D*1	1·27	250·5	84·4
*D*2	1·04	266·6	131·7
*D*3	2·52	286·1	190·4
*D*4	2·36	288·9	209·5
*D*5	3·02	291·7	212·4
*D*6	3·39	293·0	217·0
*D*7	2·57	317·1*	202·7
Total	16·17		

* Equivalents of esters (freed from unsaponifiable matter): *A*8, 298·8; *B*9, 297·6; *C*10, 315·4; *D*7, 306·3.

In the C_{18} series, spectrophotometric evidence thus obtained for the presence of linolenic and linoleic acids was confirmed by the production from the acid of ester fractions *C*7, *C*8, *D*5 and *D*6 of typical bromo-additive products: (*a*) hexabromostearic acid, m.p. 179–180° (unchanged on admixture with a specimen of authentic hexabromostearic acid from the acids of linseed oil), and (*b*) a crystalline specimen of (impure) tetrabromostearic acid which melted at 95–100°, this melting point being somewhat raised when the acid was mixed with authentic tetrabromostearic acid (m.p. 114·5°) from vegetable sources. (The effective separation of pure tetrabromostearic acid from a mixture of tetra- and hexabromostearic acids is very difficult, and the low melting point of the specimen finally obtained is almost certainly caused by traces of the hexabromo-acid still being present.)

From the analytical results in Table 2 the compositions of the respective unsaturated C_{18} or C_{16} acids present in the four fractions analysed, and from these the iodine values and equivalents of the corresponding unsaturated methyl esters, were calculated. These data were employed in calculating the composition of all the ester fractions in the respective groups *B*, *C* and *D*, it being assumed that the composition of the C_{18} (or C_{16}) unsaturated esters was approximately constant in all the distilled fractions concerned (this assumption is not completely justified, but introduces no significant error into the analyses).

The composition of each ester fraction was then evaluated by methods which have been described by Hilditch (1947, pp. 498–509), and the percentage composition (by weight) of each group of acids thus calculated, and hence the composition of the total fatty acids of the horse mesenteric fat (Table 3).

Component glycerides of horse mesenteric fat

The fat (268 g.) was submitted to systematic crystallizations from 10% solutions in acetone at temperatures successively rising from −65 to 0°. Certain intermediate fractions left in solution were combined and subjected to further crystallization from acetone at appropriate temperatures. The glycerides, which were finally insoluble in acetone at 0°, were given a further separation from solution in ether at 0°. Each solution was left at the selected temperature for 4·5–5 hr., with the exception of the crystallizations at 0°, which were left overnight in a refrigerator. A complete scheme of the separations used is given in Fig. 2.

The mixed acids in the groups A–G of the glycerides were determined by fractionation of their methyl esters, those in groups C, D and E being first separated into relatively saturated and unsaturated portions by crystallization from ether or acetone at −35°. The acids in the small groups H and I of greatest solubility and unsaturation were determined from spectrophotometric analyses alone, the small proportion of saturated acids present therein being taken as palmitic acid. Each of these analyses thus involved a series of determinations and consequently calculations of the same nature as those described above, and illustrated in Tables 1–3, for the component acids of the whole horse fat. The details of each component acid analysis are therefore

Table 2. *Spectrophotometric analyses of unsaturated* C_{16} *and unsaturated* C_{18} *acids*

Acids from fractions	I_2 value	$E\frac{1\%}{1\,cm.}$ (after alkali-isomerization) At 268 mμ.	At 234 mμ.	Composition of acids (%) Unsaturated Series	Monoene	Diene	Triene	Saturated
*B*6	95·5	23	37	C_{18}	90·1	1·3	4·3	4·3
*C*8	177·2	225	369	C_{18}	39·5	14·1	42·3	4·1
*D*6	228·0	358	570	C_{18}	6·7	20·6	67·9	5·4
*D*2	138·9	119	211	C_{16}	60·9	8·3	20·1	10·7

Reference values ($E\frac{1\%}{1\,cm.}$) used for pure di- and tri-enoic acids:

	λ (mμ.)	C_{18} acids* ($E\frac{1\%}{1\,cm.}$)	C_{16} acids† ($E\frac{1\%}{1\,cm.}$)
Trienoic acid, alkali-isomerized at 170° for 15 min.	268	532	592
Trienoic acid, alkali-isomerized at 180° for 60 min.	234	569	633
Dienoic acid, alkali-isomerized at 180° for 60 min.	234	906	1006

* Hilditch *et al.* (1945).

† Calculated from molar extinction coefficients ε, derived from C_{18} acid data.

Table 3. *Component acids in groups A, B, C and D, and in the whole horse mesenteric fat*

Acid	Component acids in the groups *A* (28·5%)* (%)†	*B* (32·1%)* (%)†	*C* (22·6%)* (%)†	*D* (16·8%)* (%)†	Fatty acids in the whole fat (%, w/w)	(%, by mol.)
Lauric (dodecanoic)	—	—	0·9	1·3	0·4	0·6
Myristic	5·8	4·6	4·4	2·2	4·5	5·3
Palmitic	69·7	10·2	7·8	5·7	25·9	27·3
Stearic	11·9	3·9	—	—	4·7	4·4
Arachidic (eicosanoic)	0·8	—	—	—	0·2	0·2
Hexadecenoic	—	4·4	9·3	9·2	5·1	5·4
Hexadecadienoic	—	—	1·2	1·2	0·5	0·5
Hexadecatrienoic	—	—	3·1	3·1	1·2	1·5
Oleic	11·6	72·1	27·9	5·0	33·7	32·2
Linoleic	—	1·1	9·9	15·4	5·2	5·0
Linolenic	—	3·4	29·8	50·3	16·3	15·8
Unsaturated C_{20-22}	—	—	5·4	6·0	2·3	2·0
Unsaponifiable	0·2	0·3	0·3	0·6	—	—

* Groups as % (w/w) of total acids.

† Component acids as % (w/w) of group.

not recorded here, owing to the space that would be required. Table 4, however, shows the proportions and characteristics of each of the groups A–I of glycerides obtained by crystallization, and also the percentage composition (by weight and by molecular proportions) determined from the detailed analysis of the fatty acids in each group of glycerides.

In considering the glyceride composition of each of the individual groups A–I, the minor proportions of lauric acid present may be taken with myristic acid, and those of arachidic with stearic acid. The increment (by mol.) of each fatty acid category in each glyceride group is shown in Table 5. Considering, in the first place, the relative amounts of saturated and unsaturated acids in the glycerides of the different groups, it is clear that the most saturated glycerides have been concentrated in the least soluble fractions A and B, which indeed contain some trisaturated glycerides in addition to mono-unsaturated disaturated glycerides. In fraction C, di- as well as mono-unsaturated glycerides are present; in view of the very small proportion of trisaturated glycerides in the preceding fraction B, it is reasonable to suppose that the latter are not present in C. In the remaining fractions, tri-unsaturated glycerides are evidently present in increasing proportions; it is possible that fraction D may also contain some mono-unsaturated glycerides, but in view of the drastic crystallization procedure applied, and of the fact that glycerides such as oleopalmitostearin are relatively insoluble in comparison with the di- and tri-unsaturated glycerides present, it is very unlikely that any appreciable proportion of mono-unsaturated glycerides have been left in fraction D, and still less so in the later fractions. Similarly, it is almost certain that no di-unsaturated glycerides have been retained in the least soluble fractions A and B. Thus, when care has been taken to pursue the original crystallization of the fat to a point at which little or no change takes place on further recrystallization of intermediate glyceride fractions, it can be assumed with considerable certainty that any one

glyceride group contains only binary mixtures of tri- and di-saturated, di- and mono-saturated, or mono-saturated and tri-unsaturated glycerides. The proportions of such binary mixtures can be calculated from the component acid data (Table 5, i).

to indicate that each will contribute only one acyl group to any triglyceride in which it is present.

By summation of the increments of acids or of glyceride categories in each of the nine groups of glycerides, the amount of each in the whole fat

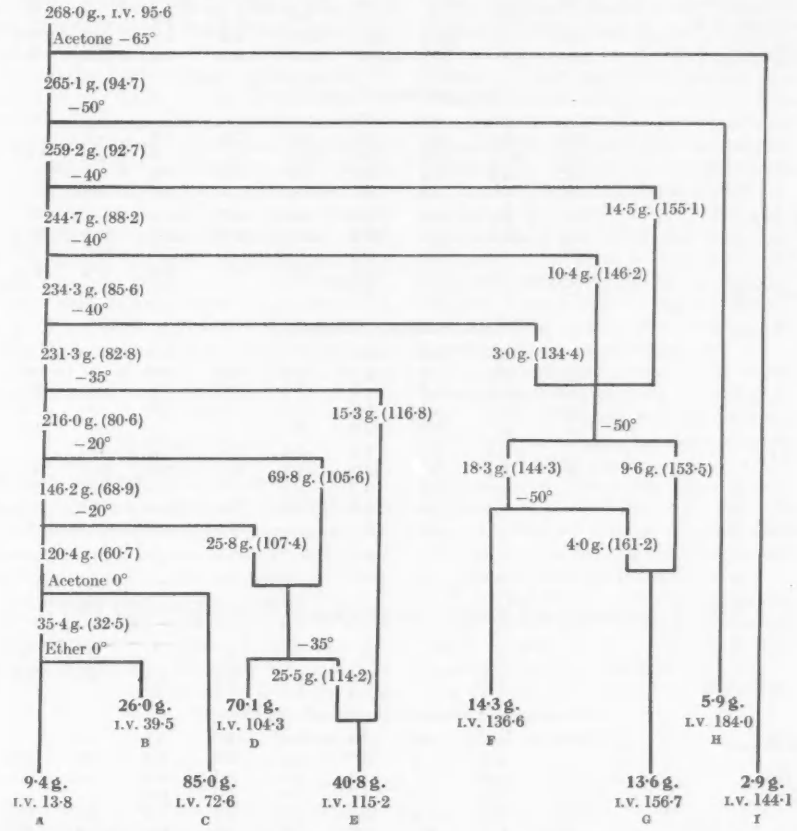

Fig. 2. Crystallization of mixed glycerides of horse mesenteric fat. All crystallizations were from 10 % solutions in acetone, except in one instance (ether) indicated. Figures in parentheses are iodine values (I.V.).

The probable proportions, in each glyceride group, of mono-, di-, and tri-palmitoglycerides (Table 5, ii), mono- and di-oleoglycerides (Table 5, iii), and mono- and di-linolenoglycerides (Table 5, iv), can be deduced on similar lines, although it should be noted that the degree of segregation of the unsaturated glycerides, at all events, cannot be postulated with so much certainty as in the case of the various categories of saturated-unsaturated triglycerides.

The proportions of the remaining acids (myristic, stearic, unsaturated C_{16}, linoleic, unsaturated C_{20-22} acids) in all the groups of glycerides are small enough

(Table 5, final column) is computed. Comparison of the fatty acid figures (mol. %) thus found for the whole fat with those for the direct analysis of its total fatty acids (Table 3) is a partial check on the degree of analytical accuracy of the component glyceride analysis: the figures are in close accordance except in the cases of palmitic and oleic acids, where the differences are respectively 1·4 and 1·8 units (mol. %).

When, in any of the groups of glycerides, the number of different fatty acids present is not more than three, it is possible by consideration of the

Table 4. *Horse fat glyceride groups obtained by low-temperature crystallization*

	A	B	C	D	E	F	G	H	I	Total
Weight (g.)	9·4	26·0	85·0	70·1	40·8	14·3	13·6	5·9	2·9	268·0
Iodine value	13·8	39·5	72·6	104·3	115·2	136·6	156·7	184·0	144·1	95·6
Saponification equivalent	270·2	276·8	281·2	287·4	290·2	287·6	288·8	295·5	350·6	283·1
Unsaponifiable (%, w/w)	—	0·2	0·1	0·5	1·4	0·6	0·8	2·4	18·5	—
Glycerides (%, w/w)	3·5	9·7	31·7	26·2	15·2	5·3	5·1	2·2	1·1	100·0
Glycerides (% by mol.)	3·7	10·0	32·1	25·9	15·0	5·3	5·0	2·1	0·9	100·0
Component acids (%, w/w)										
Lauric	—	0·6	0·5	0·3	—	—	—	—	—	0·3
Myristic	16·9	8·0	4·2	3·3	2·1	3·9	2·4	—	—	4·2
Palmitic	55·2	44·3	32·6	17·0	16·3	6·2	5·2	9·5	17·2	24·5
Stearic	12·1	13·9	5·4	4·0	1·2	1·1	2·0	—	—	4·9
Arachidic	—	—	0·4	0·3	0·5	—	—	—	—	0·2
Unsaturated C_{16}	—	2·9	6·3	6·9	4·8	10·3	10·7	—	—	6·0
Oleic	15·8	23·5	34·1	40·4	43·4	40·3	30·4	24·6	32·5	35·5
Linoleic	—	1·7	5·3	6·3	8·5	9·8	9·3	12·7	9·9	6·2
Linolenic	—	5·0	9·8	16·9	23·2	28·4	39·0	53·2	40·4	16·6
Unsaturated C_{20-22}	—	—	1·4	4·4	—	—	—	—	—	1·6
Component acids (% by mol.)										
Lauric	—	0·8	0·6	0·4	—	—	—	—	—	0·4
Myristic	19·1	9·2	5·0	4·0	2·6	4·7	2·8	—	—	5·0
Palmitic	55·5	45·6	34·1	18·2	17·4	6·6	5·5	10·3	18·5	25·9
Stearic	10·9	12·9	5·1	3·8	1·2	1·1	1·9	—	—	4·7
Arachidic	—	—	0·3	0·3	0·4	—	—	—	—	0·2
Unsaturated C_{16}	—	3·0	6·6	7·4	5·2	11·0	11·7	—	—	6·3
Oleic	14·5	22·1	32·5	39·3	42·1	39·1	29·5	24·1	31·8	34·0
Linoleic	—	1·6	5·1	6·2	8·3	9·6	9·2	12·6	9·7	6·0
Linolenic	—	4·8	9·5	16·6	22·8	27·9	38·4	53·0	40·0	16·1
Unsaturated C_{20-22}	—	—	1·2	3·8	—	—	—	—	—	1·4

Table 5. *Component acid and glyceride categories in the glyceride groups* A *to* I

	A	B	C	D	E	F	G	H	I	Total
Glycerides % (by mol.)	3·7	10·0	32·1	25·9	15·0	5·3	5·0	2·1	0·9	100·0
Component acid categories (increments, % by mol.)										
Myristic (with C_{12})	0·7	1·0	1·8	1·1	0·4	0·2	0·1	—	—	5·3
Palmitic	2·1	4·6	10·9	4·7	2·6	0·4	0·3	0·2	0·2	26·0
Stearic (with C_{20})	0·4	1·3	1·7	1·1	0·2	0·1	0·1	—	—	4·9
Unsaturated C_{16}	—	0·3	2·1	1·9	0·8	0·5	0·7	—	—	6·3
Oleic	0·5	2·2	10·4	10·2	6·3	2·1	1·5	0·5	0·3	34·0
Linoleic	—	0·1	1·7	1·6	1·3	0·5	0·4	0·3	0·1	6·0
Linolenic	—	0·5	3·1	4·3	3·4	1·5	1·9	1·1	0·3	16·1
Unsaturated C_{20-22}	—	—	0·4	1·0	—	—	—	—	—	1·4
Component glyceride categories (increments, % by mol.)										
(i) Trisaturated	2·1	0·5	—	—	—	—	—	—	—	2·6
Disaturated-mono-unsaturated	1·6	9·5	11·3	—	—	—	—	—	—	22·4
Monosaturated-di-unsaturated	—	—	20·8	20·7	9·7	2·0	1·5	0·7	0·5	55·9
Tri-unsaturated	—	—	—	5·2	5·3	3·3	3·5	1·4	0·4	19·1
(ii) Dipalmito-	2·5	3·7	0·7	—	—	—	—	—	—	6·9
Monopalmito-	1·2	6·3	31·4	14·1	7·8	1·1	0·8	0·7	0·5	63·9
No palmito-	—	—	—	11·8	7·2	4·2	4·2	1·4	0·4	29·2
(iii) Dioleo-	—	—	—	4·6	4·0	0·9	—	—	—	9·5
Mono-oleo-	1·6	6·6	31·3	21·3	11·0	4·4	4·4	1·5	0·8	82·9
No oleo	2·1	3·4	0·8	—	—	—	0·6	0·6	0·1	7·6
(iv) Dilinoleno-	—	—	—	—	—	—	0·8	1·2	0·2	2·2
Monolinoleno-	—	1·4	9·2	12·9	10·2	4·4	5·2	0·9	0·7	43·9
No linoleno-	3·7	8·6	22·9	13·0	4·8	0·9	—	—	—	53·9

glyceride categories i–iv (Table 5) to give a fairly close estimate of the individual mixed glycerides present in each group, and thus to give an approximate statement of the probable component glycerides present in the whole fat (Barker & Hilditch, 1950). In the horse fat glycerides, however, there are more than three acids present, often in fairly high proportions, in each group of separated glycerides, and consequently there are several combinations of mixed triglycerides each of which would satisfy the conditions i–iv in Table 5. It is therefore not practicable to suggest any detailed statement for all the mixed glycerides present. The major components of the horse fat studied are, however, undoubtedly palmito-oleo-linolenins (approx. 30%), and glycerides with one oleic and two other (usually different) unsaturated acyl groups (approx. 18%); the rest of the fat is made up of oleodipalmitin, oleomyristopalmitin and oleopalmitostearin (approx. 6–7% each), with lesser proportions (approx. 5% each) of palmitodiolein, palmito-hexadeceno-olein, and palmito-oleo-linolein, and still smaller amounts of a number of other mixed glycerides, including about 2% of trisaturated glycerides.

DISCUSSION

Component fatty acids of horse mesenteric fat

The composition of the mixed fatty acids (Table 3) shows that the horse fat resembles other animal body fats in many respects, but stands apart in its high content of linolenic acid and in the presence of more linoleic and unsaturated C_{16} acids than is usual in such fats. Its saturated acids are typical of those animal depot fats which are not rich in stearic acid. Palmitic acid is the main saturated acid and falls within the range of 30 ± 3% (by mol.) of the total fatty acids, which Banks & Hilditch (1931) considered to be characteristic for the fats of land animals. Myristic and stearic are the only other saturated acids present in appreciable amounts in the horse fat, but each forms only about 5% of the total fatty acids.

The unsaturated acids, apart from very small proportions of acids of the C_{20} (and possibly C_{22}) series, belong entirely to the C_{16} or C_{18} series. In the C_{16} group there are small but definite amounts of a hexadecatrienoic acid of the linolenic type, as shown by the spectrophotometric evidence after alkali-isomerization. The latter also suggests the presence of still smaller proportions of a hexadecadienoic acid, but in the absence of more conclusive evidence it is perhaps better merely to record the presence of the triene acid and to note that the mean unsaturation of the unsaturated acids is $-2{\cdot}8$H (i.e. 2·8 atoms of hydrogen/molecule short of saturation). The total amount of unsaturated C_{16} acids is definitely greater in the horse fat than in corresponding fats from, for instance, the ox, sheep or pig.

The characteristic presence of relatively large proportions of linolenic and linoleic acids (identical with these acids as found in vegetable fats) has been fully confirmed. In the present instance, linolenic acid formed 16% and linoleic acid 5% of the total fatty acids. At this point, however, it is useful to compare our present data with the other recently recorded detailed studies of horse fats to which reference was made in the introduction to this paper. The relevant data are given in Table 6.

Whilst the offal fat of Brooker & Shortland (1950) and the kidney fat of Holmberg & Rosenqvist (1949) may be considered most analogous to the mesenteric horse fat which we have examined, it will be noticed that the linolenic acid (17%) and also the linoleic

Table 6. *Component fatty acids of horse fats*

	Mesenteric*	Offal†	Bone†	Hoof†	Milk‡	Kidney§	Marrow§ (*a*)	Marrow§ (*b*)
Iodine value of fat	95·6	97·9	99·0	109·3	84·3	84·4	90·2	84·9
Component acids (%, w/w)								
Lauric	0·4	—	0·2	—	5·6‖	0·6	—	—
Myristic	4·5	1·1	3·1	0·8	7·0	2·8	2·7	4·4
Palmitic	25·9	27·4	25·3	17·9	16·1	20·3	22·0	20·0
Stearic	4·7	1·7	4·1	2·5	2·9	7·2	1·6	2·2
Arachidic	0·2	—	—	0·7	0·3	—	—	0·3
Unsaturated C_{16} (and C_{14})	6·8	11·3	8·9	19·4	9·3¶	4·3	10·9	10·9
Oleic	33·7	34·7	35·1	34·3	18·7	38·8	36·3	42·0
Linoleic	5·2	5·1	5·2	5·1	7·6	22·1	14·6	14·6
Linolenic	16·3	17·0	17·3	16·9	16·1	2·2	2·4	3·6
Unsaturated C_{20-22}	2·3	1·7	0·7	2·4	5·1	1·5	8·4	1·9

(*a*) Gelatinous marrow. (*b*) From porous bone.

* Present work.
† Brooker & Shorland (1950).
‡ Hilditch & Jasperson (1944).
§ Holmberg & Rosenqvist (1949).
‖ Also 5·5% decanoic (capric), 2·6% octanoic (caprylic), 0·9% hexanoic (caproic) and 0·4% butyric acids.
¶ Also 1·9% unsaturated C_{12} and C_{10} acids.

acid (5%) contents of all three New Zealand fats (offal, bone and hoof) closely resemble that observed by us, whereas the kidney and bone marrow fats of the Swedish horse contained much smaller proportions (2–4%) of linolenic acid, but much more linoleic acid (15–22%). On the other hand, the mare milk fat, studied by Hilditch & Jasperson (1944), also contained 16% of linolenic acid. It may be suggested that these consistent differences may depend upon the diet of the animals. Holmberg & Rosenqvist (1949) state that the fats which they examined were from a 19-year-old gelding fed entirely on hay and oats during the last months before slaughtering. The milk fat (Hilditch & Jasperson, 1949) was from a mare fed on pasture in summer and on hay with a small amount of crushed oats in winter. The New Zealand fats studied by Brooker & Shorland (1950) were from horses which were invariably pasture fed. Unfortunately we have not been able to ascertain the feed of the animal from which the fat used in the present work was derived. The data as a whole, however, are consistent with the probability that the grass fats (rich in linolenic acid) are the source of the linolenic glycerides of the horse depot fats: it is not known whether these linolenic glycerides are still present in comparable proportions in hay, but they are reported to be absent from the fatty oil of oats which, however, contains high proportions of linoleic glycerides (Amberger & Hill, 1927).

The palmitic acid content of the Swedish horse depot fats is a few per cent lower than that of the New Zealand or English fats, but apart from the differences in linolenic and linoleic acid content mentioned the mesenteric, offal, kidney and bone fats all exhibit fairly close resemblance in fatty acid composition. The content of unsaturated C_{16} acids in the English mesenteric fat is, however, somewhat lower than that in most of the other horse fats. All six fats are characterized by the presence of 34–40% of the chief component, oleic acid, and by the absence of more than minor proportions of stearic acid.

The two other fats quoted in Table 6, mare milk fat and horse hoof oil, show not only the characteristics peculiar to horse fat, but also those of the particular organ fats concerned. Thus, in linolenic and linoleic acid contents both fats are very closely similar to the intestinal depot fats from English or New Zealand horses. At the same time, the horse hoof fat has the high hexadecenoic acid and lower palmitic acid content which Hilditch & Shrivastava (1948) observed in specimens of neat's foot oil of Irish and Australian origin. Similarly, the mare milk fat has a much reduced proportion of oleic acid and, to a less degree, of palmitic acid, with a corresponding introduction of shorter chain unsaturated and saturated acids (the latter, however, not extending much below the C_{10} series). In connexion with the suggestion that the short-chain acids of milk fats may be derived from the oleoglycerides of the blood fat (Hilditch, 1947, pp. 306–310), it may be significant that, whilst the oleic acid content of the mare milk fat is only about half that of the horse depot fats, its linoleic and linolenic acid contents are almost exactly the same as those in the latter fats.

We are of the opinion that horse depot fat (and, doubtless, many other animal fats) may well represent a mixture of synthesized and assimilated dietary fat, in which polyethenoid acids present in the dietary fats have undergone little or no transformation prior to their deposition in the adipose tissues. This is a relatively simple instance of assimilation, whereas, with Brooker & Shorland (1950), we regard the ingestion of fat by ruminant animals as a particular case in which polyethenoid C_{18} glycerides may be altered during prolonged digestion, and, moreover, where oleoglycerides are also converted in part into stearoglycerides before storage in the fat depots. The fats, depot or milk, of ruminant species are indeed to be looked upon as a special instance of a more complicated character than the processes of fat assimilation and transformation in many non-ruminant animals. For the rest, our conclusions on these points are in entire agreement with the arguments put forward in some detail by Brooker & Shorland. Moreover, on the basis of different dietary conditions in the two cases, we consider that the apparently divergent results of Holmberg & Rosenqvist (1949) are quite reconcilable with the observations of the New Zealand workers and ourselves.

Component glycerides of horse mesenteric fat

Besides the three major component acids—oleic, palmitic and linolenic—which together make up 75% of the mixed fatty acids of the horse fat, five other acids each contribute about 5% to the total, with the result that a complicated mixture of mixed glycerides is present. Consideration of Table 5 shows, however, that the fat exhibits the usual tendency for its constituent fatty acids to be distributed on the whole evenly throughout the triglyceride molecules. In this respect it stands in contrast to animal fats which are rich in stearic acid and which are characterized by much more trisaturated glycerides (palmitostearins) than is consistent with the simple operation of the usual principle of 'even distribution' (Hilditch, 1949).

This principle was first observed by Collin & Hilditch (1929) in vegetable seed fats, and expressed by these authors as a 'pronounced tendency to even distribution of the fatty acids throughout the glycerides'. Subsequently it has been defined (Hilditch, 1947, p. 16) more explicitly by stating that when a given fatty acid forms about 35% or more of the total fatty acids in a fat, it will occur at least once

in practically all the triglyceride molecules of the fat; but that a simple triglyceride containing three identical acid groups does not appear until the acid in question forms about 65 % or more of the total fatty acids. Owing to the comparative simplicity of the fatty acid mixtures in many vegetable fats, the latter lend themselves more readily to investigation of glyceride constitution than the more complicated fats. Recently (Barker & Hilditch, 1950; Hilditch & Seavell, 1950) study of a large number of vegetable fatty oils has provided data which show that, for a given individual acid, the proportion of glycerides with one group of the acid reaches a maximum when the acid forms 33 % of the total acids, and the proportion of glycerides with two groups of the acids reaches a maximum when the acid forms 67 % of the total acids; but the maxima are about 80–90 % of the total glycerides in the fat, whereas rigid adherence to the 'rule of even distribution' would, of course, imply that the whole of the fat would be constituted entirely at these points of glycerides containing either one or two of the acyl groups in question. In other words, some of the glycerides commence to contain two acyl groups somewhat before the acid concerned forms one-third of the total fatty acids, and some simple triglycerides with three acyl groups of the acid concerned appear before the latter forms two-thirds of the total fatty acids. This recent work has served to define more rigidly, and to confirm, the more general statement first given by Collin & Hilditch in 1929.

The general constitution of the horse fat glycerides, in which eight or nine component acids have to be considered, can be followed by consideration of the distribution of the individual acids in the mixed glycerides, especially that of the major component acids oleic, palmitic and linolenic. Oleic forms 34 % (by mol.) of the total fatty acids, i.e. if it were wholly evenly distributed it would be present (once) in all the triglyceride molecules. Table 5 (iii) shows that actually it is absent from 8 % of the latter, and that 9 % of glycerides with two oleic groups are present in the horse fat. This is exactly similar to the proportions of dilinoleo- or dilinoleno-glycerides observed in vegetable fatty oils (Hilditch & Seavell, 1950) when the proportion of linoleic or linolenic acid is of a similar order. Similar data for stearic-rich animal fats, such as those of the ox or sheep, show that oleic acid is absent from considerably more of their triglycerides than in the present instance, and illustrate the manner in which such fats differ constitutively from the more usual type:

Depot fat	Oleic acid % (by mol.)	Glycerides containing no oleic groups % (by mol.)
Sheep (Hilditch & Zaky, 1941)	40·4	14
Ox (Hilditch & Paul, 1938)	38·7	17
Ox (Hilditch & Murti, 1940)	30·5	28

In the fats mentioned, the proportion of trisaturated glycerides is thus from 14 to 28 % of the whole, whereas the horse fat now studied contained only 2·5 % of trisaturated glycerides.

Palmitic acid, the next most prominent acid in the horse fat (of the acids of which it forms 26 % by mol.), would if strictly evenly distributed be present in all but 22 % of the triglyceride molecules. Actually it is absent from 29 % of the triglycerides, 7 % of the fat consisting of dipalmitoglycerides. Linolenic acid, which forms 16 % of the total fatty acids, occurs as dilinolenoglycerides in an extremely small proportion (2 %) of the fat. All the other acids present only occur once in any triglyceride molecule, so far as can be ascertained from our investigation of the horse fat component glycerides.

The horse fat glycerides are thus a somewhat complicated mixture of mixed glycerides, constituted on the usual lines of approximation to even distribution of each fatty acid amongst the glycerol molecules.

SUMMARY

1. The component acids of mesenteric fat from a horse consisted mainly of oleic (34 %), palmitic (26%) and linolenic (octadeca-9:12:15-trienoic)(16%) acids, with about 5–6 % each of myristic (tetradecanoic), stearic, hexadecenoic, and linoleic (octadeca-9:12-dienoic) acids, 2 % of higher unsaturated acids and traces of lauric (dodecanoic) and arachidic (eicosanoic) acids.

2. The presence of ordinary linolenic and linoleic acids in some quantity in horse fats is confirmed by the present study. The proportion of these acids observed is the same as in a group of New Zealand horse fats recently examined by Brooker & Shorland (1950), but both results differ from the experience of Holmberg & Rosenqvist (1949), who found more linoleic and much less linolenic acid in a Swedish horse fat. It is probable that the variations are connected with differences in the diet of the animals.

3. The component glycerides of the horse fat have been examined in some detail and found to consist of a complex mixture of mixed glycerides, each individual acid being for the most part distributed evenly throughout the triglyceride molecules. In general structure, therefore, the horse fat glycerides resemble those of vegetable fats, marine animal oils, etc., rather than those of other land animals, such as oxen and sheep, which are rich in stearic acid and which also contain an abnormal proportion of trisaturated glycerides (palmitostearins). The chief individual components of the horse fat include palmito-oleo-linolenins (approx. 30 %) and glycerides with one oleic acid and two other (usually different) unsaturated acyl groups (approx. 18 %), with lesser proportions of oleodisaturated glycerides, palmitodiolein, palmito-hexadeceno-olein and pal-

mito-oleo-linolein, and very small amounts of many other mixed glycerides. Trisaturated glycerides form little more than 2% of the whole fat.

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Fish Tropomyosin and Fish Nucleotropomyosin

By G. HAMOIR
Lister Institute, London, and Laboratoire de Biologie générale, Université de Liège

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Tropomyosin has been prepared from muscles of several animals (rabbit, pig, horse and whiting) by dehydrating the minced muscle with organic solvents and extracting with M-potassium chloride (Bailey, 1948). A detailed account of its isolation and properties has also been published (Bailey, 1948; Bailey, Gutfreund & Ogston, 1948; Astbury, Reed & Spark, 1948).

In the course of an extensive ultracentrifugal study on fish myosins, it has been found that two proteins showing the same solubility properties as tropomyosin can be isolated from fish muscles without previous treatment with organic solvents. Both tropomyosins have been obtained in crystalline form and their ultracentrifugal and electrophoretic behaviours have been investigated. One of these proteins is practically identical with Bailey's tropomyosin. Dr K. Bailey kindly suggested that the other, which had the larger sedimentation constant, might be an association of the first compound and nucleic acid. This was completely confirmed by phosphorus and ribose determinations and by the ultraviolet absorption. The name 'nucleotropomyosin' is proposed for this compound.

EXPERIMENTAL

Isolation

Extraction of fish muscles with salt solutions (mixtures of phosphate and potassium chloride) of varying pH between 5 and 7 shows that the proportion of tropomyosin in the extract increases when the pH of the extraction fluid decreases and when the time of extraction is short. The tropomyosins are still very extractable when the duration of the extraction is 10 min. and the pH of the extract 5·5, but the amount of myosins going into solution under such conditions becomes much smaller. The proportion of the tropomyosins in the extracts can be further increased if the extraction is carried out with muscles kept for several months in the frozen state. Such treatment decreases the extractability of the myosins (Reay & Kuchel, 1936) and does not change the behaviour of the tropomyosins. Separation of nucleotropomyosin from tropomyosin in such extracts is easy. On dilution with water the former coprecipitates with the myosins while the latter remains in solution and precipitates only on acidifying the supernatant to pH 4·6. A final purification of the substances is carried out by ammonium sulphate fractionation. The details of the method are given below.

The preparation has been carried out throughout in the cold and all the separations have been done by centrifugation. Carp muscles cut with a freezing microtome into slices 40 μ thick (Dubuisson, 1947) are extracted for 20 min. with 3 vol. of a KCl-phosphate solution (0·15 M-KCl and 0·1 M-KH_2PO_4 brought to pH 5). This extract is diluted with 3·5 vol. of cold water; the precipitate is discarded and the supernatant (I) kept.

The residue from this first extraction is re-extracted for 10 min. with 3 vol. of 0·5 M-phosphate solution of pH 5·5 containing 0·3% sodium adenosinetriphosphate. The

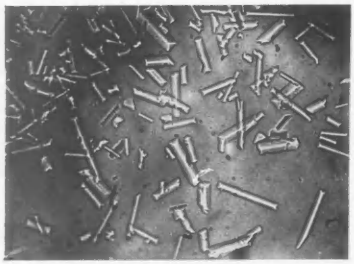


Fig. 1. Nucleotropomyosin (magnification × 54).

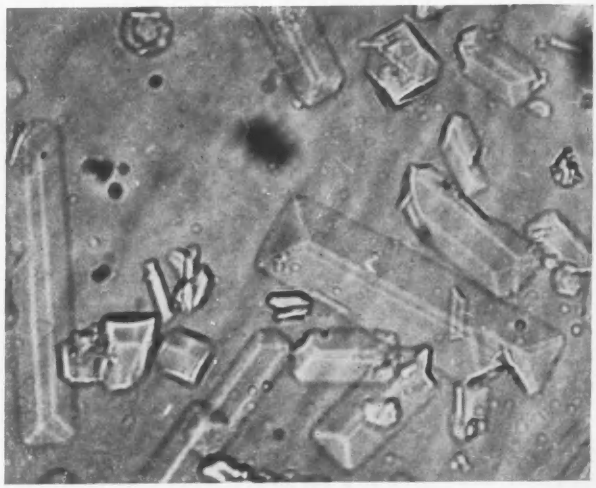


Fig. 2. Nucleotropomyosin (magnification × 135).

Fig. 3. Tropomyosin (magnification × 135).

residue is discarded and the extract (II) is diluted with 7 vol. of water. A precipitate of myosins and nucleotropomyosin forms which is washed twice with water and redissolved in 0·5 M-KCl at neutral pH. The supernatant (II) and the supernatant (I) are mixed and brought to pH 4·6. The precipitate containing tropomyosin is washed twice with water and redissolved in 0·5 M-KCl at neutral pH.

Both solutions are now centrifuged for 30 min. at 14,000 rev./min. to remove some turbid material and are purified by a second precipitation by dilution with 8 vol. of water at neutral pH (nucleotropomyosin) or at pH 4·6 (tropomyosin). Both precipitates are washed twice with water and redissolved in 0·5 M-KCl at neutral pH.

Both tropomyosins are isolated from these two solutions by $(NH_4)_2SO_4$ fractionation at neutral pH: the major part of the total protein content of the solutions precipitates between 30 and 50 % saturation, whilst the tropomyosins precipitate between 50 and 66 % saturation. The precipitate can be redissolved very quickly by a slight dilution with water, giving a water-clear solution.

Although these methods of preparation are very reproducible, some variations are observed in the yields obtained, which are usually about 0·07 % of the wet weight for nucleotropomyosin and 0·03 % for tropomyosin. A better yield, amounting to 0·5 % of the fresh muscle weight, has been obtained after organic solvent treatment (Bailey, 1948). This discrepancy is not due to loss during the isolation. The quantity of tropomyosins and myosins extracted from fresh muscles under the conditions described amounts to 1 % of the wet weight and the tropomyosin content of the solution varies from 10 to 20 %. Fractionation by $(NH_4)_2SO_4$ is very effective: no precipitation of tropomyosin occurs before 40 % saturation while the major part of the myosins has already been removed. The denaturation of the myosins by organic solvent treatment probably increases the differences in extractability of the myosins and tropomyosins and allows a more complete removal of the tropomyosins.

Crystallization. The undiluted salted-out precipitates are used for crystallization. This is carried out using the conditions previously described for tropomyosin (Bailey, 1948), i.e. by dialysing an approx. 1·5 % solution against a solution containing 16 g. $(NH_4)_2SO_4$/l. and 0·01 M-acetate buffer of pH 5·4. Nucleotropomyosin crystallizes in elongated prisms (Figs. 1, 2); tropomyosin in the quadrangular plates previously described by Bailey (Fig. 3). From a private communication of Dr Bailey it would appear that the crystalline form of nucleotropomyosin is not characteristic of this compound alone: similar crystals have already been obtained by him from fish (whiting) tropomyosin solutions prepared in the usual way.

Ultracentrifugation

The solutions were examined in the Svedberg oil-turbine ultracentrifuge using the Philpot optical system (Philpot, 1938) at 60,000 rev./min. The tropomyosins were dissolved in a phosphate buffer of ionic strength 0·1 containing 0·25 M-sodium chloride. The pH of the solution was 7·1. The concentrations of the protein were determined refractometrically, assuming a specific refractive increment of 0·00180. The values of the sedimentation constants have been corrected to 20° and to a water basis, using the partial specific volume given by Svedberg & Pedersen (1940), Appendix II.

The preparation of tropomyosin appears homogeneous by ultracentrifugation. It sediments as a single fairly symmetrical peak whatever the concentration (Fig. 4*a*), and the values of the sedimentation constants at different concentrations obtained from different preparations are in good agreement (Fig. 5 and Table 1). The sedimentation constant extrapolated to zero concentration is 2·85. The two values previously obtained for rabbit tropomyosin (Bailey *et al.* 1948) have been added to the diagram; they show excellent agreement with the present determinations.

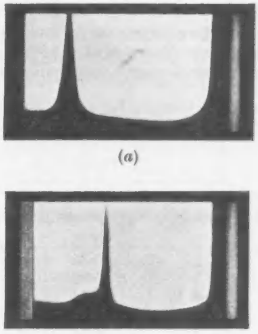


Fig. 4. Sedimentation diagram of tropomyosin and nucleotropomysin. Migration to the right. Tropomyosin (*a*): 0·89 %, 100 min. after full speed. Nucleotropomyosin (*b*): 0·67 %, 90 min. after reaching full speed.

The behaviour of the preparation of nucleotropomyosin is more complicated: it sediments as a main sharp peak which spreads much more slowly than tropomyosin and is always followed by a small component (Fig. 4*b*). The proportion of this component increases at low concentrations; it is not an impurity but a dissociation product of the main component, the sedimentation constant-concentration curve of which (Fig. 5) cannot therefore be extrapolated.

Several ultracentrifugations have been done to check the stability of nucleotropomyosin. Its sedimentation is unchanged in the presence of adenosinetriphosphate (ATP) or after keeping the concentrated solution at the pH of crystallization (5·4) for a few days. In acetate buffer of pH 3·5 nucleotropomyosin is slowly and irreversibly transformed in the cold as shown in Fig. 6: a new component sedimenting more slowly appears after 2 hr. (Fig. 6*a*) and becomes prominent after 20 hr. (Fig. 6*b*). Nucleotropomyosin as an entity disappears completely if the solution is kept a longer time at this pH. Sedimentation runs made with solutions of nucleotropo-

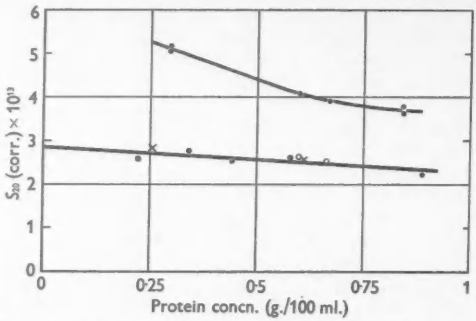


Fig. 5. Variation of S_{20} for tropomyosin and nucleotropomyosin with the concentration of the solution. Lower curve; tropomyosin; upper curve, nucleotropomyosin. ○, values taken from Bailey *et al.* (1948); ×, values obtained by transformation of nucleotropomyosin at low pH.

bigger and more asymmetrical particles of nucleotropomyosin are transformed at low pH into tropomyosin.

Electrophoresis

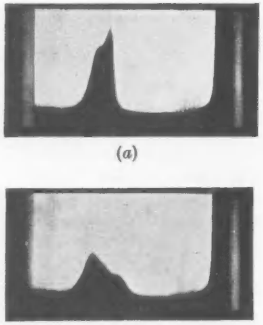
The experiments were performed with the Tiselius electrophoresis apparatus slightly modified (Dubuisson & Jacob, 1945; Dubuisson, Distèche & Debot, 1950). They lasted about 20 hr. at $I = 0{\cdot}35$ (1·65 V./cm.) and 7 hr. at $I = 0{\cdot}15$ (3·65 V./cm.), giving a migration of approx. 6 cm. in both cases. No account was taken, in the calculation of the mobilities, of the differences in conductivity of the dialysis fluid and the protein solution, the value for the dialysis fluid being used for both limbs.

Both preparations were homogeneous electrophoretically, showing a single component with no, or only a slight, admixture of impurities. No difference in mobility was observed between nucleotropomyosin and tropomyosin in several experiments made on pure solutions and on mixtures of both compounds. The mobilities found are given in Table 2.

Table 1. *Sedimentation data for tropomyosin and nucleotropomyosin at different concentrations in solutions* ($I = 0{\cdot}35$, pH 7·1.)

Tropomyosin			Nucleotropomyosin		
Sample no.	Protein concn. (g./100 ml.)	S_{20} corr. × 10^{13}	Sample no.	Protein concn. (g./100 ml.)	S_{20} corr. × 10^{13}
1	0·58	2·57	1	0·84	3·79
2	0·34	2·73	2	0·84	3·63
3	0·89	2·22		0·6	4·08
	0·44	2·52		0·3	5·11
	0·22	2·59	3	0·67	3·95
4*	0·25	2·82		0·3	5·14
5*	0·61	2·57			

* Obtained by transformation of nucleotropomyosin at pH 3·5.



(*a*)

(*b*)

Fig. 6. Sedimentation diagrams of nucleotropomyosin kept in acetate buffer pH 3·5 for 2 hr. (*a*, total concn. 1·1 %) and 20 hr. (*b*, total concn. 0·87 %); both 130 min. after reaching full speed. Migration to the right.

myosin kept 20 hr. or more in acetate buffer at pH 3·5 show that the peak corresponding to this new component has the same shape and rate of sedimentation as tropomyosin (Fig. 5 and Table 1). The

Table 2. *Electrophoretic mobilities of tropomyosin and nucleotropomyosin*

Conditions of electrophoresis	Mobility (10^{-5} sq.cm./V./sec.) Ascending value	Descending value
NaCl-phosphate buffer of $I = 0{\cdot}35$ and pH 7·1 ($I = 0{\cdot}1$ for phosphate and 0·25 for NaCl)	−4·30	−3·90
NaCl-phosphate buffer of $I = 0{\cdot}15$ and pH 7·4 ($I = 0{\cdot}1$ for phosphate and 0·05 for NaCl)	−6·90	−6·20
NaCl-acetate buffer of $I = 0{\cdot}15$ and pH 3·5 ($I = 0{\cdot}1$ for acetate and 0·05 for NaCl)	+6·15*	+5·76*

* Approximate value: one determination only.

The values obtained at $I = 0{\cdot}35$ and pH 7·1 differ greatly from the mobilities of rabbit tropomyosin

under the same conditions (ascending, 5·6; descending, 4·9: Dubuisson, 1950). Further investigations will be necessary to determine the cause of this difference.

Nucleic acid content

Liberation of the nucleic acid was performed by heating the protein solution in a boiling-water bath in 5 % (w/v) trichloroacetic acid for 45 min. The protein was removed by filtration and the ribose content of the filtrate was determined according to Mejbaum (1939), using a 45 min. heating time as proposed by Albaum & Umbreit (1947). Measurements were made with a photoelectric absorptiometer using the 607 Ilford filter with a maximum transmission at 600 mμ. Readings were referred to a calibration curve obtained by measuring the absorption of different amounts of D-ribose under the same conditions. As both deoxyribose and ribose react with the orcinol reagent of Mejbaum (1939), the diphenylamine reaction specific for deoxyribose was performed on the trichloroacetic filtrate under the conditions described by Dische (1930). The total phosphorus contents of nucleotropomyosin and tropomyosin were determined by the perchloric acid method as modified by Allen (1940), using the same photoelectric absorptiometer as for ribose and the 608 Ilford filter.

The ultraviolet spectrum of both proteins dissolved in the phosphate-sodium chloride buffer of pH 7·1 and $I = 0{\cdot}35$ was measured against the solvent with the Beckman universal spectrophotometer (model D.U.). (These solutions had already been used in the ultracentrifugal experiments.) Readings were taken at 3 mμ. intervals between 220 and 350 mμ. The concentrations of the solutions, expressed in g./100 ml., were determined by micro-Kjeldahl assuming a nitrogen content of 16·6 % (Bailey, 1948) and were used for the calculation of the extinction coefficient $E^{1\%}_{1\,cm.}$.

Both preparations were precipitated by an equal volume of 10% (w/v) trichloroacetic acid and kept in a boiling-water bath for different intervals to determine the time required for complete extraction (Table 3). (A slight liberation of nucleic acid occurs even after precipitation at room temperature.) Approximately constant values were obtained after about 20 min. for tropomyosin and 30 min. for nucleotropomyosin. A heating period of 45 min. was therefore chosen for the determinations.

Table 3. *Rate of nucleic acid liberation at* 100° *in* 5 % (*w/v*) *trichloroacetic acid*

Duration of heating (min.)	Ribose liberated (mg./g. protein)	
	Tropomyosin	Nucleotropomyosin
0	4·4	5·35
10	10·2	32·1
18	10·4	—
21	—	34·6
30	12·7	44·0
45	11·0	44·0
60	10·0	—

The ribose and phosphorus contents found are given in Table 4. In view of the very different calibration curves given by ribose and deoxyribose with the orcinol reagent (Schneider, 1945) and the good agreement observed between the ribose and phosphorus determinations, the presence of deoxyribose is very unlikely. Tests for deoxyribose carried out in both preparations of nucleotropomyosin failed to give any evidence of the presence of this sugar.

Samples of the first preparation were used for measurements of the ultraviolet absorption (Fig. 7).

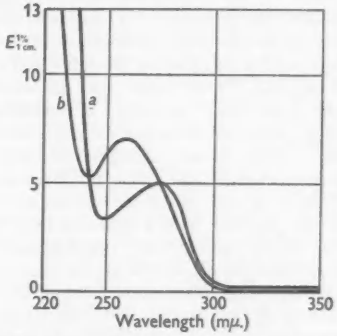


Fig. 7. Ultraviolet absorption spectra of tropomyosin (curve *a*) and nucleotropomyosin (curve *b*) in a phosphate-NaCl buffer of pH 7·1 and $I = 0{\cdot}35$. Ordinate: $E^{1\%}_{1\,cm.}$ for tropomyosin and $0{\cdot}25\,E^{1\%}_{1\,cm.}$ for nucleotropomyosin.

Nucleotropomyosin shows the well known nucleic acid maximum at about 260 mμ., and tropomyosin a maximum at 274 mμ. due to its content of aro-

Table 4. *Ribose and phosphorus contents of tropomyosin and nucleotropomyosin*

		Ribose (%)	P (%)	Ribose/P (mol./atom)	Pentosenucleic acid content* (%)
Preparation I	Tropomyosin	1·05	—	—	2·36
	Nucleotropomyosin	4·38	—	—	9·9
Preparation II	Tropomyosin	1·22	0·20	1·25	2·75
	Nucleotropomyosin	6·75	1·47	0·99	15·2

* Calculated from the ribose content.

matic amino-acids. The two spectra are very similar to those obtained by Markham, Matthews & Smith (1948) on a plant virus and its specific protein.

In conclusion, tropomyosin prepared by the present method is less pure than Bailey's tropomyosin. It contains a few per cent of pentosenucleic acid which could probably be very easily removed by keeping it for a certain time at low pH. Nucleotropomyosin has a much higher content of pentosenucleic acid, which shows some variation from one preparation to the other. The presence of pentosenucleic acid in muscle is well known from the work of Davidson & Waymouth (1944) on sheep and fowl muscles and of Schneider & Klug (1946) on rat muscles, but no analytical data seem to be available on fish. It can be assumed from the yield of 0·05–0·1 % of the fresh weight found by the previous authors for pentosenucleic acid that the amount in fish muscle is high enough to explain the present analytical results.

DISCUSSION

Comparison of the data obtained allows some conclusions to be drawn on the influence of the nucleic acid content on the macromolecular behaviour of tropomyosin. The presence of nucleic acid does not affect the electrophoretic mobility, as has already been observed for mixtures of proteins and nucleic acid (Greenstein, 1944) and for nucleoproteins (Markham *et al.* 1948). A content of nucleic acid of less than 3 % does not influence the ultracentrifugal behaviour of tropomyosin. A higher content (about 10 %) greatly changes the shape and dimensions of the molecule: a new component, named nucleotropomyosin, appears with a different rate of sedimentation which seems also uninfluenced by a variation of a few per cent in the nucleic acid content. Solutions showing an intermediate behaviour between tropomyosin and nucleotropomyosin in the ultracentrifuge were only very rarely observed.

Owing to the presence of ATP during extraction, the question arises whether nucleotropomyosin is not formed by some reaction with ATP. As it could be isolated from extracts made without addition of ATP to the extractant, and as preliminary experiments have shown that the content of ATP of fish muscles kept in a frozen state is extremely low ($\leqslant 0{\cdot}01$ % of the fresh weight), this hypothesis seems unlikely.

Nucleotropomyosin appears as a normal constituent of muscle extracts made at sufficiently high ionic strength to bring the myosins into solution. On dilution of the extract with water it is precipitated with the globulins. The phosphorus content of many myosins and actomyosins previously extracted with hot organic solvents (Dubuisson, 1941; Bailey, 1942) is probably due to the presence of nucleic acid, as already suggested by Bailey (1946) and Buchtal, Deutsch, Knappeis & Munch-Petersen (1949).

The present results confirm the view that tropomyosin is a natural component of the muscle. Further work will be necessary to know if nucleotropomyosin arises from the association of tropomyosin with pentosenucleic acid during extraction or pre-exists in fish muscle. As tropomyosin is quite probably located in the fibril (Bailey, 1948) and pentosenucleic acid is a component of the cytoplasm, it is not unreasonable to think that both compounds could be associated *in situ*.

SUMMARY

1. Two proteins showing the solubility properties of tropomyosin have been isolated from fish muscle by salt extraction.

2. Although the two compounds are indistinguishable by electrophoresis, they crystallize in different forms and behave very differently in the ultracentrifuge. These differences have been related to their different nucleic acid contents.

3. It is concluded that one compound is practically identical with Bailey's tropomyosin and the other one is a combination of the same compound with pentosenucleic acid.

4. An especially mild method of splitting this nucleoprotein is described and the occurrence of both compounds in muscle discussed.

It is a pleasure to thank Dr R. A. Kekwick for his kind hospitality and his advice, Sir Alan Drury, F.R.S., Director of the Lister Institute, for the facilities made available by him, and Dr K. Bailey for a helpful suggestion. The work was made possible by a grant from the Belgian Government.

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The Effect of Starvation on the Pseudo-Cholinesterase Activity of the Liver and Serum of Rats

By MARION F. HARRISON and LUCY M. BROWN
Medical Research Council Department of Experimental Medicine, University of Cambridge

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In man the serum cholinesterase has been found to be low in many diseases affecting the cells of the liver and to rise again to normal levels with recovery, and the measurement of the activity in the serum has been suggested as a test of liver function (McArdle, 1940; Faber, 1943; Kaswin, 1945). McCance, Widdowson & Hutchinson (1948) showed that the serum possessed subnormal activity during undernutrition, and that normal activity returned with recovery. Waterlow (1950) took liver biopsies and simultaneous samples of serum from infants during and after a period of malnutrition, and found that the enzyme activities increased in both tissues as nutrition improved.

Some have assumed that the pseudo-cholinesterase found in the serum has been made by the liver and discharged into the circulation, but the experiments undertaken by others to prove this on animals have not been conclusive. This has partly been due to a tendency for each species and sex to respond to the same stimulus in a different way, but the difficulties have been enhanced by the variable amounts of 'true' cholinesterase found in the serum and organs of the domestic and laboratory mammals. Thus chloroform and carbon tetrachloride poisoning raised the cholinesterase in the serum of dogs (Steensholt & Venndt, 1945; Brauer & Root, 1947), but ligaturing the pancreatic duct, phosphorus poisoning, or undernutrition made no permanent impression on the serum activity (Hutchinson, 1951). Brauer & Root (1947) found that the cholinesterase activity of the liver in dogs was depressed after prolonged carbon tetrachloride poisoning. In rats, carbon tetrachloride lowered the serum cholinesterase in females but not significantly in males, and it reduced the activity in the livers of both sexes (Ellis, Sanders & Bodansky, 1947; Brauer & Root, 1946). Hutchinson (1951) observed that feeding half rations for 3 months halved the weight of adult male rats, but did not lower the activity of the serum cholinesterase. On the other hand, Mendel, Hawkins & Nishikawara (1948) determined the serum cholinesterase of males before and after a 12-day fast and found that the serum activity fell by 50%. Milch (1950) also found a fall in activity after feeding a protein-free diet for 6 weeks. No similar experiments have been carried out on female rats, although other liver enzymes have been followed during starvation in both sexes (Miller, 1948).

No significant sex difference has been found in the serum cholinesterase activity of dogs (Brauer & Root, 1947), or of human beings except during pregnancy (Levine & Hoyt, 1949). In rats, however, the females have much more cholinesterase in their livers and sera than the males (Birkhäuser & Zeller, 1940; Sawyer & Everett, 1947). Both tissues contain true and pseudo-cholinesterase, but it is the latter which is responsible for the sexual variation (Mundell, 1944; Sawyer & Everett, 1946).

In the present study, the cholinesterase activity of the liver and the serum of male and female rats was followed during a 6-day period of starvation. At the same time, other changes in the composition of the liver cell during starvation were investigated by one of us (M.F.H.) and these will be reported in a later paper.

METHODS

Animals. Thirty-five male and thirty-five virgin female black and white hooded rats of the Lister strain, 3–4 months old, were used. Until the fast, they were fed on the stock diet consisting of bread, milk, whole wheat, wheat germ, yeast, fish meal, oatflakes and bran. The rats were weighed twice a week for 3 weeks before the fast to ensure that they were growing at a normal rate. The males and females were then divided into five comparable groups of seven rats, according to their mean weight for the 3 days before the fast,

and to their mean increase in weight during the 3 pre-experimental weeks. One group of each sex was killed at the commencement between 11 a.m. and 12 noon and served as controls. The remaining rats were kept in cages which were raised to prevent coprophagy. Water was given *ad lib.*, but the animals received no food of any kind. The rats were weighed daily between 10.45 and 11 a.m.; and a group was killed after 1, 2, 4 and 6 days, respectively, at the same time of day as the control group.

The rats were stunned, decapitated and samples of blood collected in tubes. The livers were removed quickly, washed for a moment in cold water, dried between filter paper, weighed and placed in a refrigerator at 0°. The livers from the seven rats were pooled and homogenized at 0° with five times their weight of water (Folley & Watson, 1948). The sera from the rats' blood were pooled by mixing an equal volume of serum from each rat in the group.

Cholinesterase. Enzymic activity was measured manometrically at 37° and pH 7·4. The substrates used were acetylcholine chloride (ACh) and benzoylcholine chloride (BCh), both obtained from Roche Products Ltd. Solutions were freshly prepared for each estimation, and the final concentrations in the flasks were 0·06 M-ACh and 0·01 M-BCh. The reaction was carried out in 0·024 M-$NaHCO_3$ in an atmosphere of 5 % CO_2 and 95 % N_2. Sufficient enzyme was used to give an evolution of 50–200 μl. CO_2/30 min. The production of CO_2 by the tissues in the absence of substrate was trifling and in consequence has been neglected. Readings were taken 3 min. after adding the substrate and then at 10 min. intervals for 30 min., during which time the evolution of CO_2 was linear. All estimations of enzymic and non-enzymic hydrolysis were carried out in duplicate. The results were corrected for non-enzymic hydrolysis, and the unit of cholinesterase was defined as the amount of enzyme which liberated 1 μl. CO_2/min. from acetylcholine.

Total serum proteins and serum albumin were separated as described in Laboratory Techniques (1951).

The 'protein N' of the liver was obtained by adding ice-cold trichloroacetic acid to a sample of the homogenate and estimating the N in the precipitate. All determinations of N were made by the micro-Kjeldahl method (Hawk, Oser & Summerson, 1947).

Note. Mendel, Mundell & Rudney (1943) stated that ACh in a concentration of 0·06 M was not hydrolysed by the true cholinesterase. If this is true the ratio of the rate of hydrolysis of BCh to the rate of the hydrolysis of ACh should be constant for all tissues of the same animal unless each tissue has its own characteristic pseudo-cholinesterase. In fact, the ratios varied and were as follows: average for sera, males, 0·14; females, 0·22; for livers (male), 0·19. The ratio for the livers of females up to the second day was 0·32, whereas that from the second to sixth day was only 0·26. The way in which these ratios differ suggests that when the activity of the pseudo-cholinesterase was small the true enzyme may have made an appreciable contribution to the hydrolysis of ACh. The results have been given as the rate of hydrolysis of ACh, and the difference between male and female would have been greater than it appeared to be if some correction for the contribution of the true cholinesterase had been made.

RESULTS

The results are summarized in Table 1. It will be seen that initially the male rats were heavier than the

Table 1. *The pseudo-cholinesterase and 'protein N' content of the liver of male and female rats after fasting for* 1, 2, 4 *and* 6 *days*

(Standard deviations of the mean weights are given in brackets below the mean value.)

		Body weight		Liver weight		'Protein N'		Cholinesterase units	
Sex	Length of fast (days)	Initial (g.)	At death (g.)	Total (g.)	Per 100 g. initial body wt. (g.)	Per g. liver (mg.)	Per 100 g. initial body wt. (mg.)	Per g. liver	Per 100 g. initial body wt.
Male	0	270 (24)	270 (24)	8·91 (1·49)	3·30 (0·40)	31·4	104	29	96
	1	272 (18)	256 (19)	7·03 (0·38)	2·59 (0·18)	38·4	100	32	83
	2	271 (21)	244 (19)	6·70 (0·54)	2·47 (0·08)	37·6	93	32	79
	4	271 (22)	229 (18)	5·80 (0·59)	2·14 (0·20)	37·6	80	30	64
	6	271 (23)	210 (20)	5·41 (0·58)	2·00 (0·10)	37·3	75	35	70
Female	0	208 (16)	208 (18)	6·20 (0·61)	2·98 (0·18)	32·0	95	111	330
	1	209 (17)	200 (15)	5·13 (0·50)	2·46 (0·21)	36·0	89	72	178
	2	209 (15)	189 (16)	4·67 (0·22)	2·25 (0·12)	37·0	83	49	110
	4	208 (15)	180 (15)	4·39 (0·28)	2·11 (0·13)	36·7	78	53	112
	6	208 (16)	167 (14)	4·44 (0·34)	2·13 (0·12)	33·4	71	45	96

female rats, the mean weights being 271 and 208 g., respectively. During the fast the body weights fell continuously, and after 6 days the males had lost 61 g. and the females 41 g., equivalent to a loss of 23 and 20% of their initial body weights, respectively. The livers of males averaged 8·91 g. in weight before the fast, of females 6·20 g. During the fast the liver weights fell by 3·50 and 1·76 g.; the livers lost weight most rapidly during the first 24 hr. of starvation.

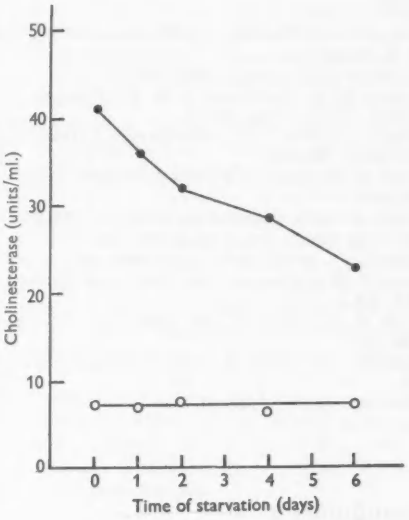

Fig. 1. Effect of starvation on the serum cholinesterase of rats. ○—○, male; ●—●, female.

The 'protein N', expressed in mg./g. liver tissue, did not change much during the fast, although there was a slight rise during the first day which was probably due to the loss of glycogen. There was a striking sex difference in the cholinesterase activity of the liver. The activity per g. liver tissue was initially 3·8 times greater in the females than in the males. In the males the activity changed little during the fast, but in the females the cholinesterase fell rapidly during the course of the first 2 days to less than half its original value and thereafter changed little.

The total amounts of cholinesterase and 'protein N' in the livers have also been expressed per 100 g. initial body weight. These figures bring out the fact that the males had relatively, as well as absolutely, more liver protein than the females and that starvation reduced it by about the same extent in both sexes. In the males the cholinesterase fell with the 'protein N' to about 71% of its initial value, but in the females there was a much greater fall in activity.

In Fig. 1 are shown the concentrations and variations in the activity of cholinesterase of the pooled sera during the 6 days fast. It will be seen that the sex difference was even greater in the serum than in the liver, the serum of females being almost six times more active than that of males. During the fast, the mean serum cholinesterase in the females fell continuously from 41·2 to 22·9 units/ml., but in the males remained constant at 7·1 units/ml. The protein decreased gradually from 7·18 to 6·75 g./100 ml. in the males, and from 7·68 to 6·68 g./100 ml. in the females, and the serum albumen from 3·78 to 3·40 g./100 ml. in the males, and from 3·88 to 3·44 g./100 ml. in the females. There was no correlation with the serum cholinesterase.

DISCUSSION

These results, taken in conjunction with those of Birkhäuser & Zeller (1940), Sawyer & Everett (1946, 1947), Ellis *et al.* (1947) and Brauer & Root (1947) show that the liver of the female rat has a very high cholinesterase activity, that this activity is maintained by the ovarian hormones, and that the greater part of it disappears when the normal functions of the liver have been disturbed by poisoning or by fasting for 2 days. Initially, the liver of the male has a much lower cholinesterase activity, but by the end of the second day of fasting the difference has largely been eliminated. In the male rat, and in the female after the second day of starvation, the cholinesterase of the liver falls per 100 g. initial body weight, but it does not change much per mg. 'protein N' in the liver. In the female there would appear to be in the liver a considerable cholinesterase activity which is labile, sex-linked and in some way different from the small basal activity which is common to both sexes and is closely associated with the liver proteins.

The fall in the serum cholinesterase of the female rat is consistent with the hypothesis that the serum activity depends on the liberation of the enzyme from the liver. It resembles that obtained in human undernutrition and liver disease, but nothing is known at present about the hormonal control of the cholinesterase activity of the liver in man, or whether there is a large labile element in it. The results for male rats are in keeping with those of Hutchinson (1951) who worked with the same colony of rats, and it is possible that those of Mendel *et al.* (1948) and Milch (1950) who obtained a fall in the serum of males, may have been due to the use of a different strain. However, it is clear that the cholinesterase activities of the liver and serum in male rats are much more stable than in females, and, like the serum enzyme in the dog, are much less easily influenced by undernutrition.

SUMMARY

1. The amount of pseudo-cholinesterase in the livers of adult male rats decreased from 96 to 70 units/100 g. initial body weight during a 6-day fast, but the activity per g. liver changed very little, and the activity of the serum enzyme remained constant at 7·1 units/ml. serum.

2. The total pseudo-cholinesterase in the livers of female rats amounted to 330 units/100 g. initial body weight. Two days' starvation reduced this figure to 110, and 6 days' starvation to 96. There was little change per g. liver after the second day. The activity in the serum fell from 41 to 23 units/ml. in the 6 days.

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Studies in Carotenogenesis

1. GENERAL CONDITIONS GOVERNING β-CAROTENE SYNTHESIS BY THE FUNGUS *PHYCOMYCES BLAKESLEEANUS* BURGEFF

BY G. A. GARTON,* T. W. GOODWIN AND W. LIJINSKY
Department of Biochemistry, The University of Liverpool

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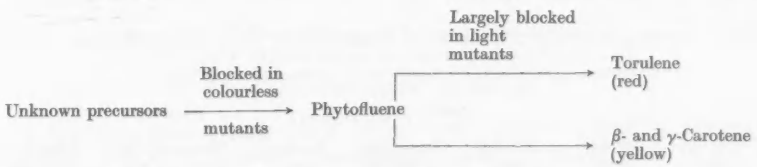
Information concerning carotenogenesis in both Phanerogams and Cryptogams is limited, and that which does exist is often far from unequivocal (see Goodwin, 1951, for a full discussion). In order to simplify the experimental approach as much as possible by eliminating photosynthesis, it was decided to investigate carotenogenesis in a fungus; *Phycomyces blakesleeanus* was an obvious choice, for it is easily cultured on simple aqueous media, requiring only aneurin as a growth factor (Schopfer, 1934), and produces considerable amounts of a single carotenoid, β-carotene (Schopfer & Jung, 1935; Karrer & Krause-Voith, 1947; Albrecht, 1948; Bernhard, 1948) together with traces of α-carotene Karrer & Krause-Voith, 1947; Albrecht, 1948; Bernhard, 1948) and, possibly, lycopene (Albrecht, 1948; Bernhard, 1948); no xanthophylls have ever been detected. *Phycomyces blakesleeanus* is a heterothallic fungus existing in two well defined strains, (+) and (−) (Blakeslee, 1904). According to Schopfer (1935), cultures of the (+) strain are generally yellower than those of the (−) strain, an observation which suggests that the (+) strain produces the greater amount of carotene.

The only quantitative information concerning carotenoid formation in *Phycomyces* is that obtained by Schopfer (1935); he records the carotene concentration as being about 0·09% on dry weight.

* Present address: The Rowett Research Institute, Bucksburn, Aberdeenshire.

Further information is restricted to short statements by Schopfer (1934) in a paper mainly concerned with other aspects of the biochemistry of *Phycomyces*. He noted that the C:N ratio of the medium was an important factor, and that glycine and asparagine were about equally effective in promoting carotenogenesis, but ammonium nitrate was better than either; further, he claimed that, when cultured in the dark or in red light, the fungus produces no β-carotene whilst in daylight or violet light it does.

Equally meagre is the information available concerning carotenoid synthesis by other fungi. Glycerol is the most effective single source of carbon for carotenogenesis by *Rhodotorula sanniei*; a mixture of lactic acid and glucose is equally effective, but glucose alone does not support pigment formation (Fromageot & Tchang, 1938). Light is apparently necessary for the biosynthesis of carotenoids by *Neurospora sitophila* (unpublished work of Deventer, quoted by Zechmeister, 1944), but is not necessary in the case of *N. crassa*, although it does stimulate production (Haxo, 1949). Bonner, Sandoval, Tang & Zechmeister (1946), in a study of carotenoid synthesis by seven ultraviolet mutants of *Rhodotorula rubra*, found that in light-coloured mutants synthesis of torulene (Lederer, 1933) was considerably restricted whilst that of the other carotenoids, including the colourless polyene phytofluene (a possible precursor of carotenoids), was unchanged; in the colourless mutants all carotenoids including phytofluene disappear. On the basis of these observations Bonner *et al.* (1946) put forward the following tentative metabolic scheme:



In order to have a firm foundation on which to base future work, a full investigation has been carried out on the general factors concerned with carotenogenesis by *Phycomyces*. A short account of part of this work has already been reported (Garton, Goodwin & Lijinsky, 1950).

EXPERIMENTAL

Cultural methods. Phycomyces blakesleeanus Burgeff, (+) and (−) strains, were obtained on malt agar slopes from the Centraalbureau voor Schimmelcultures, Baarn. They were maintained on malt agar, subcultured every 2 months and stored in the dark at room temperature. In the first part of the investigation both (+) and (−) strains were used, but later only the (−) strain, which produces more carotene, was used. Except in experiments involving medium changing, the cultures were grown in 8 oz. medicine bottles laid flat and containing 15 ml. of the appropriate liquid medium to which 3 ml. of a spore suspension had been added from an automatic pipette. The bottles were plugged with non-absorbent cotton wool and the plugs covered with cellophan caps. In medium-changing experiments 250 ml. Erlenmeyer flasks were used instead of medicine bottles.

Spore suspensions were made by taking a mature medicine-bottle agar slope (1 month old; 15 ml. of agar) adding 150 ml. of distilled water, shaking vigorously and decanting through a plug of glass wool into the reservoir of the automatic pipette. All operations were carried out under sterile conditions. The cultures were incubated in glass cabinets maintained at 25°, the temperature of optimum growth of *Phycomyces*. The cabinets received illumination during the normal hours of daylight. The glass cabinets were constructed by removing the sides from obsolete incubators and replacing them by plate glass. In order to maintain an even temperature throughout the cabinet, it was found necessary to put a small electric fan into the base of the cabinet; the electrical circuit was so arranged that when the heaters were on the fan revolved quickly and when they were off it revolved slowly. This arrangement was quite effective and even when the cabinets were full (100–120 bottles) a temperature difference greater than 2° between the top and bottom of the cabinets was rarely encountered. In one cabinet the heaters were 60 W. lamps encased in metal containers and in the other General Electric Company 60 W. enclosed heater units.

Sterilization of apparatus was carried out at 15–20 lb. pressure for 15 min. Media were sterilized either in the same way or by filtering through a Pyrex SF. 3A. porosity 5/3 filter.

The standard culture medium used was that recommended by Schopfer (1934), containing glucose (10%), L-asparagine (0·2%), $MgSO_4.7H_2O$ (0·05%), KH_2PO_4 (0·15%) and aneurin (0·025 mg. %), (all w/v), made up in glass-distilled water. Appropriate experimental media were made up as required by varying the glucose concentration, altering the main C source, or altering the N source. The amino-acids and glucose (A.R.) used were obtained from British Drug Houses Ltd. and the other sugars from T. Kerfoot and Sons; the inorganic salts were of A.R. grade.

Chemical determinations. The fungus was harvested by shaking a culture bottle until the mycelium had formed a compact mass; this was then drawn out of the bottle with a small spatula and transferred to a small Hirsch funnel attached to a water pump. The medium was then filtered through the same funnel to collect any small fragments of mycelium which might have become detached from the main mycelial mat. Total dry weight, ether-extractable lipids and β-carotene were determined on each sample in the following manner. The mycelial mat, dried as much as possible by suction, was transferred to a mortar and the lipids and β-carotene extracted by grinding under successive portions of ether freshly redistilled over reduced iron. The combined

Table 1. *The production of dry weight, lipids and β-carotene by the (−) strain of* Phycomyces blakesleeanus

(Cultured on Schopfer's medium (10% (w/v) glucose) in normal daylight at 25°. The amounts recorded are those produced in one 8 oz. medicine bottle lying horizontally and containing 15 ml. of medium.)

Time after inoculation (days)	Total dry wt. (mg.)	Lipids		β-Carotene		
		Amount (mg.)	Percentage of dry wt.	Amount (μg.)	Percentage of total dry wt.	Percentage lipids
1						
2	11·2	Trace	—	5	0·045	—
3	48	8	16·7	17	0·035	0·212
4	68	14	20·1	40	0·059	0·286
5	67	16	23·9	92	0·137	0·575
6	89	21	23·7	93	0·105	0·443
7	79	20	25·3	125	0·158	0·625
8	89	18	20·3	141	0·158	0·783
9	88	16	18·2	127	0·144	0·794
10	76	13	17·1	103	0·136	0·794
11	88	14	15·9	116	0·132	0·828
12	100	18	18·0	100	0·100	0·556
13	91	16	17·6	122	0·134	0·763
14	94	19	20·2	102	0·109	0·537
15	101	15	15·0	103	0·102	0·688
16	82	16	19·5	87	0·106	0·543
17	95	15	15·8	97	0·102	0·646
18	99	17	17·1	96	0·097	0·564
19	108	20	18·5	96	0·089	0·480
21	106	22	20·8	64	0·060	0·291
22	91	13	14·3	79	0·087	0·608
23	96	20	20·8	62	0·065	0·310
24	105	17	16·2	57	0·054	0·335
26	99	16	16·2	45	0·046	0·281
27	96	19	19·8	30	0·031	0·158
28	90	13	14·4	34	0·038	0·261
30	126	22	17·5	43	0·034	0·195
35	103	17	16·5	30	0·029	0·176
Means (6–35 days)	**95**	**18**	**19**			

Table 2. *The production of dry weight, lipids and β-carotene by the (+) strain of* Phycomyces blakesleeanus

(Grown under the same conditions as those outlined in Table 1.)

Time after inoculation (days)	Total dry wt. (mg.)	Lipids		β-Carotene		
		Amount (mg.)	Percentage of dry wt.	Amount (μg.)	Percentage of total dry wt.	Percentage of lipids
2	19	—	—	4	0·021	—
3	55	18	33	11	0·020	0·061
4	78	18	23	42	0·054	0·233
5	86	22	26	76	0·088	0·346
6	82	20	24	58	0·071	0·290
7	80	22	27	83	0·104	0·377
8	84	13	15	60	0·071	0·461
9	77	13	17	58	0·075	0·447
10	72	20	28	79	0·110	0·395
13	85	18	21	60	0·071	0·333
15	93	14	15	31	0·033	0·222
16	81	15	19	45	0·056	0·300
17	97	19	20	56	0·058	0·295
19	93	22	24	85	0·091	0·386
21	88	17	19	26	0·030	0·153
26	114	17	15	33	0·029	0·194
27	110	22	20	58	0·053	0·264
35	96	16	17	31	0·032	0·194
Means (5–35 days)	**90**	**18**	**20**			

extracts were filtered through a tared G4 sintered-glass crucible and the ether-insoluble fraction then transferred quantitatively to the crucible with the aid of a further small volume of ether. The extract containing lipids and β-carotene was reduced in volume by distillation at normal pressure in dim light on a gently boiling water bath; the concentrated extract (about 10–20 ml.) was transferred quantitatively to a tared 25 ml. Erlenmeyer flask, the remaining solvent removed in a stream of N_2, and the weight of the lipid residue determined with as little delay as possible in order to eliminate the possibility of the destruction of β-carotene. The lipid residue was then dissolved in an appropriate volume of light petroleum (A.R., b.p. 40–60°), a few drops of absolute ethanol added, and the β-carotene determined by measuring E450 mμ. in a Beckman photoelectric spectrophotometer. No chromatographic removal of the small amounts of α-carotene always present (not more than 5%) was carried out for routine analysis. The dry weight of the ether-extracted thallus was obtained by drying it in the sintered crucible at 80° for 2 hr.

Chromatographic separation of the carotenoids in Phycomyces. The lipids were extracted as already described, saponified and the unsaponifiable matter obtained using the procedure general in this laboratory (Goodwin & Morton, 1946). The residue was dissolved in light petroleum (b.p. 40–60°) and chromatographed on alumina (Spence, Grade O), slightly deactivated with methanol (Goodwin & Taha, 1950). On developing the chromatogram with light petroleum (40–60°) containing 1% (v/v) acetone, two coloured and one colourless zones were obtained, the latter being detected by its greenish-blue fluorescence in ultraviolet light.

The most weakly adsorbed zone was yellow and always constituted about 5% of the total carotenoids present. Its chromatographic behaviour suggested that it was α-carotene and this was confirmed by measuring its absorption spectrum in a number of solvents; the spectra in all cases were in accord with those reported for authentic α-carotene. The main yellow-orange zone was identified as β-carotene by its absorption spectrum and by a mixed chromatogram with an authentic specimen of crystalline β-carotene. The colourless fluorescent zone has not yet been investigated in detail, but by rechromatographing on $CaCO_3$ it could be resolved into a number of bands. The possible importance of these fluorescent substances as precursors of carotenoids must not be overlooked. It should be noted that it was not possible to detect lycopene in our extracts, although Albrecht (1948) and Bernhard (1948) considered that it probably occurred in their extracts.

RESULTS

The carotenoids in Phycomyces. It has been confirmed that β-carotene is the predominating pigment, although α-carotene also occurs in small amounts; no xanthophylls or lycopene were detected.

Production of dry weight, lipids and β-carotene under standard conditions (Schopfer's medium). A large number of experiments have been carried out to determine the dry weight, lipid and β-carotene production by the (−) strain of *Phycomyces* as a function of time and the mean values obtained are recorded in Table 1. It will be seen that maximum growth and lipid production occur within 5–6 days of inoculation, but that maximum carotene production does not occur until a few days later (7–9 days). During the ageing of the culture no change in dry weight or fat occurs, though about 14–16 days after inoculation β-carotene begins gradually to disappear from the mycelium.

Similar experiments using the (+) strain of the fungus showed exactly the same pattern, with the exception that the β-carotene level only reached about one-half that of the (−) strain (Table 2).

Table 3. *The production of dry weight, lipids, and β-carotene by the (+) and (−) strains of* Phycomyces blakesleeanus *grown in complete darkness*

(Conditions, otherwise standard, outlined in Table 1.)

Time after inoculation (days)	Total dry wt. (mg.)	Lipids		β-Carotene		
		Amount (mg.)	Percentage of dry wt.	Amount (μg.)	Percentage of total dry wt.	Percentage of lipids
(−) Strain						
4	65	13	18·5	14	0·022	0·117
6	80	13	16·3	31	0·039	0·238
7	127	29	22·8	74	0·058	0·255
8	86	14	16·3	87	0·104	0·622
9	87	14	16·1	76	0·087	0·543
13	93	19	20·4	64	0·069	0·337
16	112	23	20·5	41	0·037	0·178
19	106	15	14·1	55	0·052	0·367
Mean (6–19 days)	**99**	**18·1**	**18·3**			
(+) Strain						
7	92	10	10·9	16	0·017	0·0160
9	93	16	17·2	23	0·025	0·0144
13	101	22	21·8	25	0·025	0·0114
16	122	24	19·7	26	0·021	0·0108
Mean (7–16 days)	**102**	**18**	**17·4**			

Table 4. *The effect of varying light conditions on the growth, lipid production, and β-carotene production by* Phycomyces blakesleeanus

Conditions	Time after inoculation (days)	Total dry wt. (mg.)	Lipid (mg.)	β-Carotene (μg.)
Culture bottles wrapped in red cellophan (Fig. 1)	10	108	19	120
Culture bottles wrapped in green cellophan (Fig. 1)	10	106	16	126
Culture bottles wrapped in colourless cellophan	10	104	21	136
Cultured for 4 days in dark and then transferred to light	7	105	24	110
Cultured for 4 days in light and then transferred to dark	7	89	19	126

Role of light in β-carotene production. When grown in the dark the dry weight and fat production by both strains of *Phycomyces* were indistinguishable from those obtained with cultures kept in the light. In both cases, however, the β-carotene production was only one-half of that produced in cultures grown in the light (Table 3).

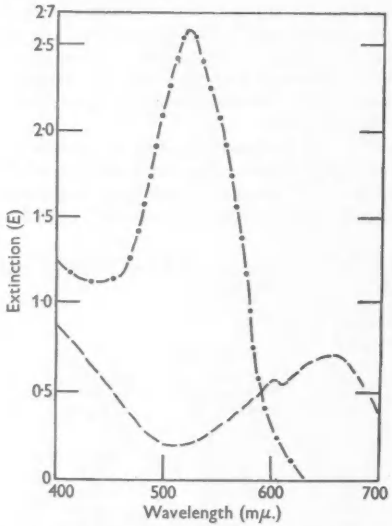


Fig. 1. Absorption spectra of the cellophans used in covering the culture bottles. The *E* values are those obtained using a single thickness of cellophan. – – –, green cellophan; –·–·–, red cellophan.

In order further to investigate the action of light, cultures were grown in bottles wrapped in either red, green or colourless cellophan. The coloured cellophans exhibited absorption spectra with maxima at 520 mμ. (red) and 605 and 660 mμ. (green), respectively (Fig. 1); The colourless cellophan reduced the intensity of light equally over the range 400–700 mμ. to the extent of 8–10 %. Table 4 indicates that as long as the fungus is exposed to light, it produces normal amounts of β-carotene irrespective of the wavelength of the illumination. This is confirmed by the observation that no changes in β-carotene synthesis were noted in cultures grown in normal daylight in June and December.

A further experiment, also recorded in Table 4, indicated the stimulatory action of light. Cultures grown in the dark for 3 days, and thus containing very little β-carotene, when subsequently placed in the light for 7 days produced an amount of β-carotene very similar to that produced during the same period by a culture grown in light all the time. Conversely, when cultures kept in daylight for 3 days are placed in the dark they continue to produce β-carotene at the 'daylight' rate.

Variation in N *source in the culture medium.* The results obtained with *Phycomyces* cultured on media containing nitrogenous compounds other than L-asparagine (Table 5) show that of the amino-acids investigated glycine, L-valine and L-leucine produced normal amounts of dry weight and lipids. Dry-weight production was normal with L-alanine, but there was a slightly lowered fat production. Glycine, however, produced more β-carotene than the other amino-acids, which produced the same amounts as did L-asparagine. Growth on L-isoleucine was poor, all three constituents being produced in amounts about one-eighth of that in control cultures. When the organism was cultured on inorganic N sources, production of dry weight and β-carotene followed a normal pattern, but fat synthesis was reduced on ammonium acetate, whilst on ammonium nitrate growth ceased when all three constituents had reached about one-quarter or one-fifth the normal values.

Some experiments were carried out using well-established mycelia, which were transferred to a medium containing no source of nitrogen, but which contained all the other constituents of the control medium. 3-Day old mycelial mats were produced

Table 5. *Dry weight, lipid and β-carotene production by* Phycomyces blakesleeanus *grown on different nitrogen sources, but under otherwise identical conditions*

(See Table 1 for growth on asparagine.)

Time after inoculation (days)	Total dry wt. (mg.)	Lipids (mg.)	β-Carotene (μg.)	Total dry wt. (mg.)	Lipids (mg.)	β-Carotene (μg.)	Total dry wt. (mg.)	Lipids (mg.)	β-Carotene (μg.)	Total dry wt. (mg.)	Lipids (mg.)	β-Carotene (μg.)
	Glycine			Alanine			Valine			Leucine		
3	32	5	9	52	6	13	28	9	24	22	3	8
4	48	10	38	70	14	42	—	—	—	—	—	—
5	49	11	42	—	—	—	—	—	—	—	—	—
6	92	22	122	80	13	86	79	18	96	28	4	24
8	65	11	149	89	13	112	—	—	—	—	—	—
10	95	17	194	96	12	90	—	—	—	—	—	—
11	97	19	168	93	10	101	—	—	—	—	—	—
12	115	22	201	—	—	—	95	24	80	84	23	111
13	93	19	145	97	12	84	—	—	—	—	—	—
18	110	28	79	101	14	73	100	19	90	96	26	98
22–24	102	17	81	93	11	65	94	9	88	74	17	76
Mean (8–24 days)	**97**	**20**		**91**	**13**		**92**	**17·5**		**85**	**22**	
	Isoleucine			NH_4NO_3			Ammonium acetate					
3	28	4	3	19	5	18	—	—	—			
4	—	—	—	19	4	19	72	10	19			
5	—	—	—	22	5	35	69	10	26			
6	18	5	9	23	5	37	80	14	59			
8	—	—	—	21	3	34	82	10	59			
10	—	—	—	22	3	25	—	—	—			
11	—	—	—	22	3	28	91	16	104			
12	11	3	16	19	2	25	—	—	—			
13	—	—	—	23	5	17	90	9	89			
18	19	7	15	27	4	25	90	10	82			
22–24	22	10	19	22	4	9	—	—	—			
Mean (8–24 days)	**16**	**7**		**22**	**3·5**		**85**	**10**				

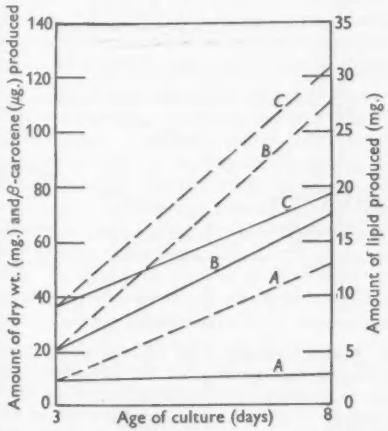


Fig. 2. Dry weight, lipid, and β-carotene production by *Phycomyces* in the presence and absence of nitrogen. – – –, on N-containing medium; ——, on N-free medium. *A*, β-carotene; *B*, lipid; *C*, dry weight.

in the normal way and then the medium was removed, the mat carefully washed with sterile distilled water and the N-free medium added: control experiments were carried out in which the normal medium was removed and replaced by a fresh sample of the same medium. The results are recorded graphically in Fig. 2. It will be seen that mycelia which are not growing, but are only dissimilating glucose, increase in dry weight and fat content, but do not produce any carotene. Whether this type of experiment is applicable to substrates other than glucose is under investigation.

Table 6. *β-Carotene production by* Phycomyces *grown on different carbohydrate sources* (3%), *but under otherwise identical conditions*

(The dry weight and lipid production are not recorded because in each case they are indistinguishable from those obtained using glucose.)

Time after inoculation (days)	β-Carotene produced (μg.)			
	Maltose	Fructose	Xylose	Glucose
4	6	13·5	4	40
6	42	27	26	93
10	111	62	49	103
13	122	57	69	96
18	96	61	74	77
23	69	45	64	62

Variation in carbon source. The ability of various carbon compounds to replace glucose was investigated. Lactose and glycerol did not support growth, whilst mycelia grown on maltose, fructose and xylose produced dry weights and lipid contents indistinguishable from the values obtained with glucose. The production of β-carotene was, however, quite definitely reduced using fructose or xylose, but was normal with maltose.

Variation in the C:N *ratio of the medium.* Two experiments were carried out to observe the effect of changing the C:N ratio of the medium on the growth, lipid and β-carotene production of *Phycomyces*. (The C value used is the total carbon in the medium, i.e. that from glucose and amino-acid.) In the first experiment the N concentration was kept constant (0·2 % L-asparagine) and the glucose concentration varied and in the second the glucose was kept constant (2·5 % glucose) whilst the N concentration was altered; the mycelia were harvested and examined at intervals from 4 to 25 days after inoculation. The effect of altering the C:N ratio can most clearly be demonstrated diagrammatically; in Figs. 3 and 4 mean values for the dry weight, lipids and β-carotene for 6–20-day-old mycelia are plotted against the C:N ratio of the medium. It will be seen that in the first experiment (N constant) (Fig. 3) dry weight increases directly with increasing C:N ratio until a value is obtained which cannot be improved by increasing the glucose concentration of the medium. Lipid production increases rather slowly at first with increasing C:N ratio and then accelerates until a maximum value is obtained at a ratio of 25:1. β-Carotene increases only very slowly until a ratio of 15:1 is reached; between 15:1 and 25:1 there is a remarkable increase (about 400 %) in production. When the glucose content is constant and the N concentration so altered to obtain the same C:N ratios as those used in the previous experiment, the results obtained were markedly different (Fig. 4). Apart from the culture containing 0·1 % of L-asparagine,

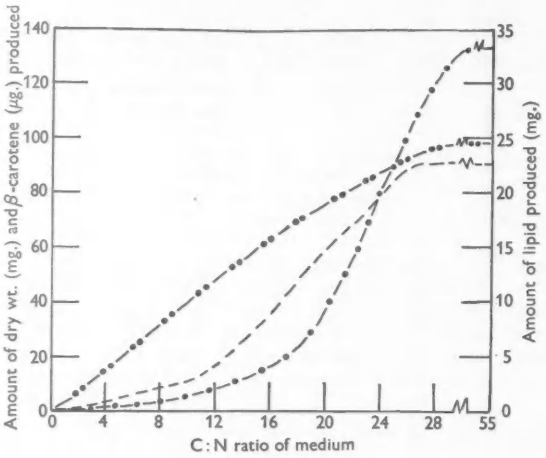


Fig. 3.

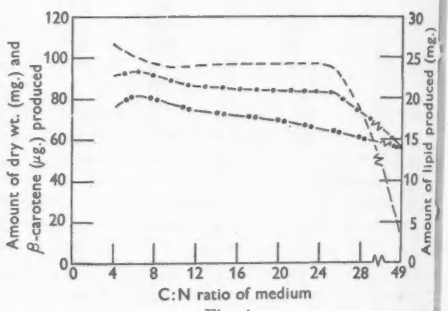


Fig. 4.

Fig. 3. The variation in the production of dry weight, lipids, and β-carotene by *Phycomyces* cultured on media containing 0·2 % (w/v) of L-asparagine and varying amounts of glucose. –·–·–, β-carotene; – – –, lipid; ·····, dry weight.

Fig. 4. The variation in the production of dry weight, lipids, and β-carotene by *Phycomyces* cultured on media containing 2·5 % (w/v) of glucose with varying amounts of L-asparagine. – – –, β-carotene; ·····, lipid; –·–·–, dry weight.

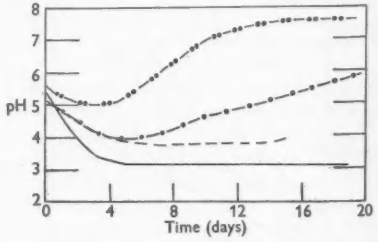


Fig. 5. The pH-time curve for different media on which *Phycomyces* was growing. – – –, Schopfer's medium (high C:N ratio); –·–·–, Schopfer's medium, but concentrations of glucose and L-asparagine altered to give medium C:N ratio; –·–·–, as above, but with low C:N ratio; ——, Schopfer's medium with L-asparagine replaced by NH_4NO_3.

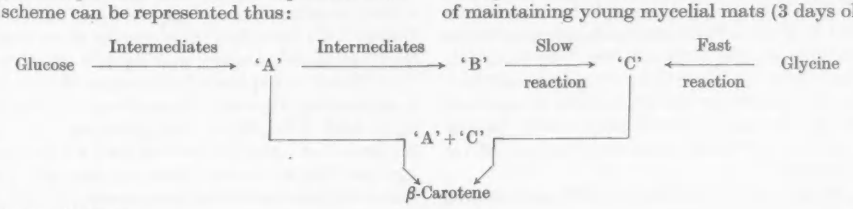
the dry-weight, lipid and β-carotene production was essentially the same for all C:N ratios. The differences between these two experiments have important theoretical implications and are discussed later.

Variations in pH *of culture media.* During growth of the mycelium the pH of the medium undergoes changes and these differ according to the type of medium in use. Changes in pH observed with the media employed in this investigation are recorded in Fig. 5. For brevity, if the changes observed with a certain medium were indistinguishable from those of the control medium, they are not recorded. It will be seen that, in most cases, the pH drops from its initial value of about 5·0 to about 3·7. In other cases, following a preliminary drop, a rise is observed in the pH which at times can reach a value of 8·0: only cultures utilizing ammonium nitrate as N source ever drop below pH 3·5.

DISCUSSION

The results reported in this paper show that, on the medium recommended by Schopfer (1934), β-carotene formation is much slower in the early stages of growth than is the production of lipids and dry weight, but after lipid and dry-weight values have reached their maxima, β-carotene production continues for some days, reaching a maximum concentration of about 0·14 and 0·7 % on dry weight and lipids, respectively (cf. Schopfer's value of 0·09 % on dry weight). In ageing cultures, the pigment gradually disappears, although the fat and dry weight remain constant. The reason for this disappearance of carotene is unknown, but the possibility of the presence of an active lipoxidase should not be overlooked.

With the various nitrogen sources examined, carotene concentration, based on either dry weight or lipid, was the same in all cases except with glycine; this amino-acid specifically stimulated carotenogenesis, producing a mycelium containing about 0·2 % of dry weight (or 1·0 % of lipid) of β-carotene. The full explanation of this must await further work, but the carbon residue remaining after the deamination of glycine must be an extremely active intermediate; further, it must be a compound which can be produced from glucose only relatively slowly; unless this were so the overwhelming preponderance of glucose in the medium (10 % compared with 0·2 %) would preclude this effect. A possible metabolic scheme can be represented thus:



The fact that glycerol is the most effective carbon source for carotenoid production in *Rhodotorula* must be taken at the moment to indicate different anabolic requirements of the fungi rather than two separate routes for carotenoid synthesis. Failure of *Phycomyces* to produce β-carotene on glycerol is primarily a failure of the fungus to grow on this carbon source.

It is significant that L-valine, which could theoretically yield β-methylcrotonic acid (a suggested intermediate), does not stimulate carotenogenesis under the present conditions. This does not rule out β-methylcrotonic acid as an intermediate, but does indicate that if it is an intermediate it can be easily formed from glucose.

It is interesting to note a differential β-carotene production in the (+) and (−) strains, the (−) strain producing, on the average, almost twice as much as the (+) strain. This observation does not agree with that of Schopfer (1935), who found the (+) strain to contain more carotene, an observation in agreement with an earlier report on carotenoids in the (+) and (−) strains of *Mucor hiemalis* (Chodat & Schopfer, 1927); whether this differential distribution has any reproductive significance remains to be seen.

Our results concerning the action of light on carotene production are also at variance with those of Schopfer (1934, 1943). He reported no pigment formation in the dark or in red light; in the present investigation experiments in which all possible precautions were taken to exclude light completely, it was found that 'dark' cultures always produced carotene to about one-half the extent of that produced by 'light' cultures, and that maximum production was achieved as soon as visible light of any wavelength was allowed to fall on the cultures. The stimulation of synthesis appears to be permanent, for 'light' cultures transferred to the dark continue to produce β-carotene at the usual rate. These observations agree with those of Haxo (1949) who found that light was not essential for carotenoid production in *Neurospora crassa*, but that it had a stimulatory effect. The observation that only half the normal amount of β-carotene is produced in the dark strongly suggests that there may be two synthetic routes, one of which is photochemical.

In order to differentiate between carotenoid production during normal growth from production during dissimilation of a carbon substrate, the effect of maintaining young mycelial mats (3 days old) on

nitrogen-free media was observed. The results (Fig. 2) indicate that with glucose as sole source of carbon, no β-carotene is formed although fat and dry weight are produced: apparently the fungus must metabolize an exogenous nitrogen source before β-carotene synthesis from glucose can take place.

It has been stated without any convincing experimental proof that the C:N ratio of the medium is the governing factor in carotenogenesis in *Phycomyces* (Schopfer, 1943) and in algae (Chodat, 1938). The present results (Figs. 3 and 4) show that under certain conditions the C:N ratio can assume a spurious significance. As long as there is sufficient nitrogen present to allow maximal growth, the C:N ratio is not important; what is important is the amount of assimilable carbon available, i.e. under most conditions, the concentration of glucose in the medium. This is well illustrated in Fig. 4; the amount of glucose present is always 2·5% (an amount just sufficient to produce maximal β-carotene production when the asparagine concentration is 0·2%), but reducing the C:N ratio by increasing the nitrogen concentration has no significant effect on carotene synthesis, or for that matter, on lipid or dry-weight production. From the pH changes in the medium it appears that the excess amino-acid is deaminated and the ammonia excreted into the medium. In this case the C:N ratio of the medium has no meaning, because it is quite different from the 'physiological' C:N ratio which can be considered as being the ratio of the amounts of carbon and nitrogen incorporated into the mycelium. Similar pH changes occur when the C:N ratio is low and the nitrogen is kept constant (Fig. 5); again excess nitrogen is discarded, but insufficient carbon is present to stimulate accumulation of carotenoid. An important point emerges from Fig. 4, namely that the values for dry weight and β-carotene obtained at a very high C:N ratio (33:1) are all low. In this case the concentration of L-asparagine was insufficient to produce maximal growth (under our conditions a fully developed mycelium contains about 4·7 mg. of nitrogen; 15 ml. of 0·2% L-asparagine provides slightly more than necessary (5·6 mg.), but 0·1% L-asparagine is inadequate). All possible growth with the amount of nitrogen available was achieved, and, as glucose was in excess, the maximal concentration of β-carotene (as opposed to amount) was achieved.

It is thus clear that the idea that the C:N ratio of a medium is the most important factor in carotene production, is of no fundamental significance in the case of *Phycomyces*, although the 'physiological' C:N ratio may be important. Provided sufficient nitrogen is present for the production of maximal growth, β-carotene synthesis only occurs to any great extent when the glucose concentration is above a certain level.

Dry-weight and lipid production. Although the main purpose of this investigation was a study of carotenogenesis, sufficient information has been obtained on the production of dry weight and lipids to make it possible to compare our results with other published data; only relative values can be compared, for varying cultural conditions may considerably affect absolute production. Bernhard & Albrecht (1948) have previously reported on the fat content of *Phycomyces* and their values, which range from 17·9 to 21·5%, are comparable with those reported here.

Concerning the effect of varying the nitrogen source on the dry-weight production, it was found that ammonium nitrate and isoleucine promoted only about 20% of the growth obtained with either L-asparagine, glycine, L-valine, L-leucine or ammonium acetate, all of which had similar growth-promoting abilities. Albrecht (1948) found L-alanine and ammonium acetate to be 82 and 61%, respectively, as effective as L-asparagine; the difference between his figure and ours for ammonium acetate is outside expected variations; at the moment only one explanation can be put forward to account for this. Albrecht harvested his cultures after 5 or 10 days; our experience indicates that growth on ammonium acetate is slower than on asparagine, although it eventually reaches the same level; thus Albrecht's ammonium acetate cultures may not have been fully grown. A further interesting result of Albrecht's work is that growth on DL-leucine is only 26% of that on L-asparagine. It might be considered that the difference between our values using L-leucine and those using DL-leucine occurred because of the better utilization of the L-isomer; however, this explanation appears unlikely for, according to Albrecht, DL-asparagine is 88% effective. Turning to Schopfer's (1934) results, we agree with him in observing that L-asparagine and glycine are equally effective, whilst ammonium nitrate is much inferior. Leonian & Lilly (1940) also found L-asparagine and glycine equally effective. The poor growth-promoting activity of ammonium nitrate is probably due to pH changes; for the first 2–4 days after inoculation, growth is normal, but thereafter it ceases; this coincides with a drop in pH below the usual value of 3·7 to 2·9.

Dry-weight production obtained using various carbon sources differs considerably in this investigation from that recorded by Schopfer (1934). The relative growths obtained by Schopfer on glucose, xylose, maltose, fructose, glycerol and lactose were 100:92:147:226:62:4. Our results show that the first four carbon sources were equally effective, and it is difficult to explain why Schopfer obtained such a stimulation of growth on maltose and fructose. We agree with Schopfer in finding lactose a very poor medium, but again differ from him with regard to glycerol, for we could obtain no mycelial growth when this was the sole carbon source.

SUMMARY

1. It has been confirmed that the major carotenoid of *Phycomyces blakesleeanus* Burgeff is β-carotene; small amounts of α-carotene are also present, but no trace of lycopene or xanthophylls was evident.

2. The dry-weight, lipid (ether-soluble) and β-carotene production by the (+) and (−) strains of *Phycomyces* has been examined under a variety of conditions.

3. On Schopfer's (1934) medium, maximal production of dry weight and lipid occurred after 5–6 days and of β-carotene after 5–9 days. β-Carotene, but not dry weight or lipid, decreased in ageing cultures. The (−) strain produces twice as much β-carotene as does the (+) strain, although lipid and dry-weight production is the same in both.

4. Light has no effect on lipid and dry-weight production; but β-carotene production is twice as great in cultures exposed to light as in those kept in the dark. When cultures exposed to light are placed in the dark they continue to produce β-carotene at the 'light' rate.

5. Substituting either glycine, L-alanine, L-leucine, or ammonium nitrate for L-asparagine has no effect on dry weight, and little on lipid production; glycine, however, specifically stimulated β-carotene production. Growth is poor on L-isoleucine and ammonium nitrate. In the absence of nitrogen from the medium no β-carotene is formed, although glucose is being metabolized.

6. When the nitrogen source is kept constant (0·2% L-asparagine), variation in the glucose concentration of the medium affects β-carotene synthesis to a much greater extent than it affects dry-weight and lipid production, but little variations are noted when nitrogen is varied and glucose kept constant (2·5%). The C:N ratio appears to be less important in controlling β-carotene production than was previously thought.

7. Substituting either xylose, fructose or maltose for glucose had no effect on lipid or dry-weight production; β-carotene production was normal with maltose, but reduced with xylose or fructose. Neither lactose nor glycerol supports mycelial growth.

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The Effect of *cis*-Vaccenic Acid on Respiration and Growth of *Bacillus subtilis*

By H. LASER
Molteno Institute, University of Cambridge

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It is well established that long-chain unsaturated fatty acids are essential for the growth of certain micro-organisms (see recent reviews by Pollock, 1949; Kodicek, 1949). With some strains of lactobacilli (Williams & Fieger, 1946, 1947; Williams, Broquist & Snell, 1947) and with *Clostridium sporogenes* (Shull & Peterson, 1948; Shull, Thoma & Peterson, 1949) oleic and similar acids are growth promoting only in absence of biotin, which they effectively replace, while with other organisms absence of biotin is not a necessary condition. However, the same acid which at a certain concentration is growth promoting becomes inhibitory at only a slightly higher concentration. This is the so-called 'double action' (Williams *et al.* 1947). The basis for the growth promotion by fatty acids and their mode of action are not yet entirely understood. Experimental evidence seems to indicate that fatty acids are metabolized 'involving an enzyme system' (Williams & Fieger, 1946), or that they 'form an essential constituent of the cell protoplasma' (Pollock, Howard & Broughton, 1949). Opposed to this view is the physico-chemical theory, that fatty acids act by being adsorbed on or incorporated into the cell surface. They are assumed thereby to change the permeability of the cell membrane towards substrates and to cause either stimulation or inhibition of growth, according to the quantitative relationship between fatty acids and preformed inhibitors of fatty acids, which are present in the cell wall (Kodicek & Worden, 1945; Kodicek, 1949). The two theories may not be mutually exclusive.

In a recent series of papers (Laser & Friedmann, 1945; Laser, 1948, 1949) a number of normal and pathological tissues have been shown to contain an unsaturated fatty acid possessing very high haemolytic activity *in vitro*. Its relation to haemolysis and to certain aspects of malaria has previously been examined (Laser, 1946, 1948, 1949). The acid has been identified by Morton & Todd (1950) as *cis*-vaccenic acid, $CH_3.(CH_2)_5.CH=CH.(CH_2)_9.COOH$, and this has been confirmed by synthesis. In the present paper the effect of *cis*-vaccenic acid on growth and respiration of *Bacillus subtilis* has been examined with a view to studying its mode of action and gaining some insight into the nature of the double effect of unsaturated fatty acids on bacteria.

MATERIAL AND METHODS

The strain of *B. subtilis* used was No. 2586 of the National Collection of Type Cultures. It was grown in Roux flasks upon the following medium: 10 g. peptone, 5 g. Lemco, 5 g. NaCl, 25 g. agar and tap water to 1 l., adjusted to pH 7·6 with NaOH and sterilized for 20 min. at 110°. The flasks were inoculated from a stock spore culture preserved on the same medium. To obtain vegetative forms, the flasks were incubated at 30° for 16 hr., when the organisms were carefully removed and washed three times with distilled water in a centrifuge.

Yeast extract was prepared by heating baker's yeast with an equal amount (w/v) of distilled water for 10 min. at 90° and filtering hot through kieselguhr. The extract was filled into ampoules, which were sterilized by steam for 30 min. and sealed, the same extract being used for all experiments described in this paper.

The medium in which the washed cells were suspended was 0·15 M-K_2HPO_4, which was obtained by adjusting the different components of the medium (cell suspension, vaccenic acid, glucose and yeast extract) in such a way as to establish a final ratio of $KH_2PO_4:K_2PO_4 = 1:1$, containing 1% glucose ± 10% yeast extract (v/v).

The fatty acids tested were synthetic *cis*- and *trans*-vaccenic acids (Morton & Todd, 1950). They were dissolved with heating in 0·3 M-K_2PO_4 (0·64 mg./ml.) and were pipetted into the reaction vessel immediately before adding the organisms, following a procedure previously described (Laser, 1949). A $1:10^5$ concentration of acid = 35·5 μM.

Growth determinations were made turbidimetrically by means of the Spekker absorptiometer with a combination of two filters (Ilford neutral H 503 and red 608). A calibration curve was prepared from serial dilutions of a washed cell suspension, the dry weight of which had previously been determined.

Growth in a liquid medium in presence of vaccenic acid was obtained in test tubes in a water bath at 30° with fairly vigorous aeration of the medium. Each tube contained a narrow capillary reaching to the bottom of the tube and connected at the upper end to a glass manifold through which water-saturated air was passed by means of a pump. A trace of tri-*n*-butyl citrate (1 drop of a fine emulsion in water) was added to each tube to prevent frothing. The initial level of liquid in the tubes was marked in order to note evaporation, and was readjusted before making density determinations.

Treatment of *B. subtilis* with vaccenic acid prior to determination of growth or O_2 uptake of treated cells was done in the same way as for the measurement of growth in the liquid medium.

Respiration was measured manometrically in Barcroft-Warburg manometers at 37°. As a rule all components of the

medium (total volume 3 ml.) were filled into the main compartment of the flasks before the beginning of the experiment, vaccenic acid being added immediately before the organisms. The initial amount of cells in all experiments was 2·4 mg. dry weight, contained in 0·1–0·2 ml. of a suitably diluted washed suspension, the density of which had been determined. In a few experiments in which the acid was added to the cell suspension at $t=0$, or in which a second amount of acid was added during the course of the experiment, flasks having two side bulbs were used. One of these contained vaccenic acid dissolved in 0·15 M-K_3PO_4 and the other an equivalent amount of 0·15 M-KH_2PO_4. The contents of the side bulbs were run into the main compartment in quick succession, KH_2PO_4 being run in first. The gaseous phase contained air or O_2. Q_{O_2} values of growing cells were calculated on the basis of the mean increase of density with time obtained in four growth experiments.

Symbols and abbreviations. $a:c$ = ratio of weight of vaccenic acid in the medium : dry wt. of cells; treated and untreated cells = cells which prior to the experiment had been incubated in presence or absence of vaccenic acid; treated and untreated supernatant = supernatant fluid from treated or untreated cells.

RESULTS

The effect of pH. The potassium salt of vaccenic acid, like that of any other fatty acid, is water-soluble at alkaline pH. It was not desirable to add the acid to the organisms in ethanolic solution, as a lipid solvent might produce, or in combination with the acid accentuate, phenomena unrelated to the acid alone. The highest pH was therefore determined at which O_2 uptake and growth of the organisms did not differ from that obtained in 0·15 M-phosphate buffer solution at pH 7·3. This was done by measuring the O_2 uptake of the organisms in presence of glucose and yeast extract in media having different ratios of KH_2PO_4:K_3PO_4, i.e. ratios of 1·6 (pH 7·3), 1·4, 1·2, 1·0 (this constituting dibasic phosphate, pH 9·2) and 0·8 : 1·0. No difference in O_2 uptake and logarithmic increase in O_2 uptake with time, corresponding to growth, was found down to the ratio $KH_2PO_4:K_3PO_4 = 1:1$. This ratio was therefore employed throughout the present experiments.

Effect of cis-*vaccenic acid on growth.* Fig. 1 shows the relation between different amounts of acid and growth based on opacity measurements. It will be seen: (1) that the acid, except for the lowest concentration in which it was used, inhibits growth; (2) that the inhibition increases with increasing amounts of acid; and (3) that the organisms recover from this inhibition and eventually attain a rate of growth at least equal to that of the control. The proportionality of the duration of inhibition of growth of the same amount of cells to the concentration of acid in the medium indicates that the governing factor is the ratio of acid : cells. That this is the case is borne out in the experiment summarized in Fig. 2, which records the relative growth rate of different amounts of organisms suspended in the same volume of medium having the same concentration of acid, i.e. varying ratios $a:c$. Here the inhibition was found to be directly related to the ratio $a:c$.

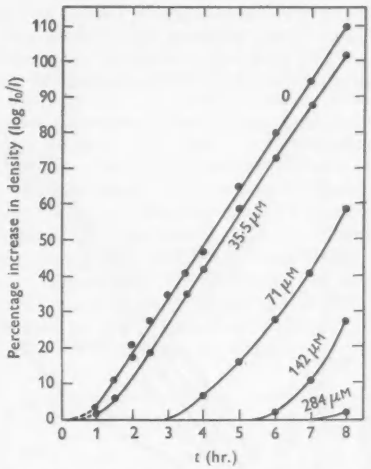

Fig. 1. Relation between varying concentrations of *cis*-vaccenic acid and growth. Initial amount of cells 2·4 mg. dry wt./3 ml. 0·15 M-K_2HPO_4, containing 1% glucose +10% yeast extract.

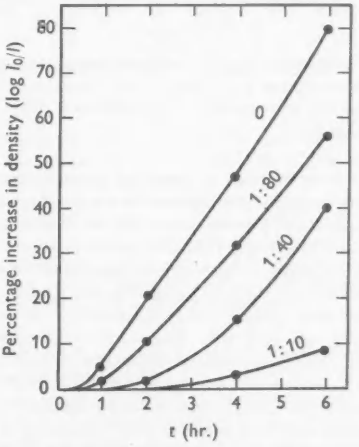

Fig. 2. Effect of varying ratios of *cis*-vaccenic acid : cells on growth. 0·15 M-K_2HPO_4, 1% glucose, 20% yeast extract. Concentration of acid in medium 142 μM, with 1·2, 4·8 and 9·6 mg. dry wt. cells/3 ml. medium; ratios of acid : cells, 1 : 10, 1 : 40 and 1 : 80.

Effect of cis-*vaccenic acid on the respiration of growing cells.* The O_2 uptake of *B. subtilis* was found to be independent of the O_2 tension between 20 and

100% O_2. Experiments in which the rate of O_2 uptake was comparatively small, as with washed cells, were therefore done in air, while those using growing cells and of longer duration were done in O_2, in order that the decreasing O_2 tension should not eventually become a limiting factor. The concentrations of acid previously tested for their effect on growth were used (Fig. 3). The decrease in total O_2 uptake was similar to that of growth, but less marked: only at the highest concentration of acid used was there a pronounced effect. However, the inhibition is greater than the additive curve of O_2 uptake suggests, and becomes apparent if the rate of O_2 uptake/mg. (Q_{O_2}) is plotted against time, i.e. if the different growth rates in presence of varying amounts of acid are taken into consideration (Fig. 4). The inhibition of respiration is not instantaneous, as there is no significant difference in the curves for the first 20 min. After that a fairly strong inhibition occurred with all concentrations of acid, the degree of inhibition being greater with increasing amounts of acid. This lasted for 20 min. only with the three lower concentrations of acid, after which gradual recovery took place, the organisms with the smallest amount of acid (35·5 μM) attaining the rate of the control after about 150 min. and remaining at that level, while the cells with an acid concentration of 71·0 and 142 μM exceeded the rate of the control by between 40 and 50% after about 180 min. The inhibition by 284 μM, on the other hand, increased after the first 20 min. and continued for 180 min. when recovery set in.

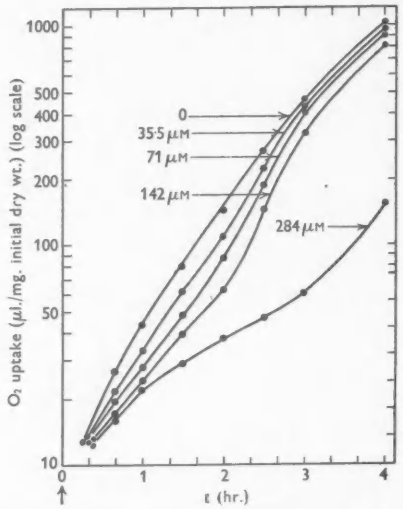

Fig. 3. Effect of *cis*-vaccenic acid on O_2 uptake with varying concentrations of acid acting on the same amount of cells (initial dry wt. = 2·4 mg.). Conditions as in Fig. 1. Acid added at $t = 0$.

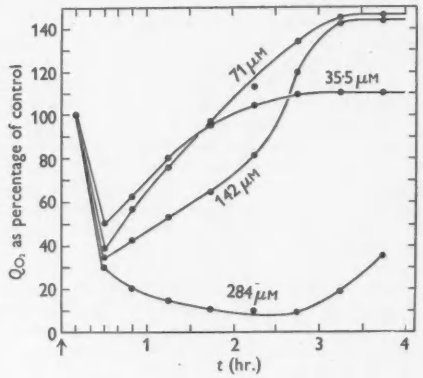

Fig. 4. Effect of *cis*-vaccenic acid on O_2 uptake, plotted as percentage of Q_{O_2} of the control in absence of acid. Acid added at $t = 0$.

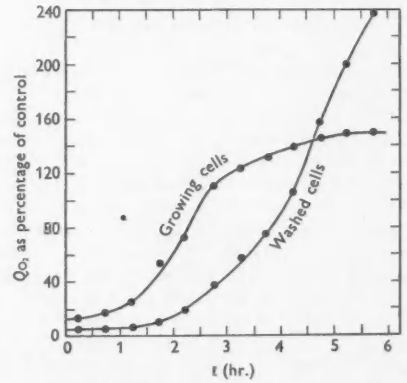

Fig. 5. Comparison of effect of *cis*-vaccenic acid on O_2 uptake of washed and growing cells, plotted as percentage of Q_{O_2} of the corresponding controls in absence of acid. 0·15 M-K_2HPO_4, containing 1% glucose ± yeast extract; 142 μM-vaccenic acid.

Effect of cis*-vaccenic acid on the respiration of washed cells.* The O_2 uptake of washed cell suspensions of *B. subtilis* in presence of glucose was found to be inhibited in a manner similar to that of growing cells and recovery from the inhibition occurred after various periods of time, according to the concentration of acid. In comparison with growing cells, the onset of recovery was usually more delayed. However, the delay was not always so marked as shown in Fig. 5, which compares the recovery from the same concentration of acid (142 μM) in presence and

absence of yeast extract. There was, however, a difference in the rate of O_2 uptake attained by either growing or washed cells after recovery from the respiratory inhibition. The Q_{O_2} of growing cells was usually stabilized at between 40 and 50% in excess of the untreated control, while that of washed cells showed an increase of between 100 and 300% over that of untreated washed cells. No growth occurred in the washed cell suspensions. The increase in O_2 uptake cannot be caused by the oxidation of the acid itself, because it was many times greater than could be accounted for by the oxidation of the amount of acid, which had been added.

Late effect of cis-*vaccenic acid.* The increased rate of O_2 uptake of *B. subtilis* following upon an inhibition of respiration by vaccenic acid continues for a considerable time. Thus cells which had been incubated with *cis*-vaccenic acid (532·5 μM, *a*:*c* = 1:20) in presence of glucose ± yeast extract had as high a Q_{O_2} after 16 hr. as immediately after recovery from the inhibition. This applied to both washed and growing cells.

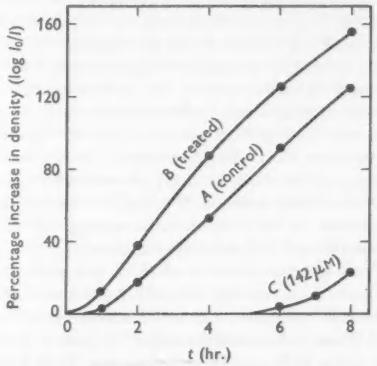

Fig. 6. Effect of incubation with *cis*-vaccenic acid on subsequent growth. Pre-incubation 16 hr., after which turbidity determinations were made and amounts equivalent to 2·4 mg. (dry wt.) transferred into fresh media.

Media: Curve *A*, pre-incubation: 1% glucose, 10% yeast extract; then 1% glucose, 10% yeast extract. Curve *B*, pre-incubation: 1% glucose, 10% yeast extract, 533 μM-vaccenic acid; *a*:*c* = 1:20; then 1% glucose, and 10% yeast extract. Curve *C*, pre-incubation: 1% glucose, 10% yeast extract; then 1% glucose, 10% yeast extract, 142 μM-vaccenic acid. *a*:*c* = 1:20.

A stimulating effect on the growth rate of treated cells, comparable to that on the Q_{O_2}, has not been established with certainty under the present experimental conditions, i.e. in a medium not deficient in biotin, although in about half the experiments a small increase of the growth rate has been observed following 16 hr. incubation with *cis*-vaccenic acid (Fig. 6). However, the fact that the slope of the growth curves after the second hour is practically the same for treated and untreated cells suggests that the observed increase is probably the result of a shorter time lag before the onset of appreciable growth. The figure shows, furthermore, that incubation and growth of cells in alkaline phosphate does not by itself produce resistance to the acid.

Repeated addition of cis-*vaccenic acid.* Since the inhibition of growth and respiration of the organisms by *cis*-vaccenic acid is transient, it was of interest to study the effect of a second addition of acid to cells which had recovered. Fig. 7 shows that when *cis*-vaccenic acid was added to cells 4·25 hr. after the beginning of the experiment, when the treated cells had re-attained a respiratory rate equal to that of the untreated control, the second addition of acid had no effect on the steady rate of O_2 consumption nor, by implication, on the growth rate, as shown by the logarithmic increase of the O_2 uptake.

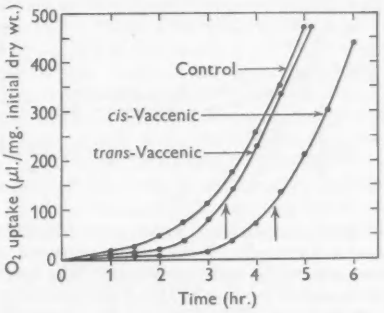

Fig. 7. Effect of second addition of vaccenic acid on O_2 uptake. Initial amount of cells 2·4 mg. dry wt./3 ml. 0·15 M-K_2HPO_4, containing 1% glucose + 10% yeast extract. Initial concentration of acid in medium 142 μM and ratio of acid:cells = 1:20. At the times indicated by vertical arrows 142 μM-*trans*- and *cis*-vaccenic acid respectively were added; *a*:*c* = 1:14 for *trans*-vaccenic and 1:10·5 for *cis*-vaccenic acids (calculated on the increase of cells at the moment of second addition of acid.)

The resistance of treated cells to further addition of acid was also found not to be restricted to the period immediately following recovery. Thus washed and growing cells which had been incubated for 16 hr. in presence of vaccenic acid (532·5 μM; *a*:*c* = 1:20) and glucose ± yeast extract, and had then been transferred into fresh media ± yeast extract and ± vaccenic acid (142 μM; *a*:*c* = 1:20), fully maintained their increased rate of respiration on renewed exposure to vaccenic acid. The growth of cells, which after recovery from the first addition of vaccenic acid (532·5 μM; *a*:*c* = 1:20) were transferred into a new medium containing yeast extract ±

vaccenic acid, was similarly unaffected by renewed contact with acid in the ratio $a:c = 1:20$.

Effect of supernatant fluid from treated cells. It seemed of interest to examine whether supernatant fluid of treated cells affects the oxidation rate of untreated cells. It was found that under the experimental conditions ($a:c = 1:20$) practically the total added acid is adsorbed by the bacteria. After short incubation at 37° or even standing at room temperature the added acid could almost quantitatively be recovered from the spun down bacteria, according to a method of extraction previously described (Laser, 1949) and determined by the haemolytic activity of the extracted acid, while the supernatant contained

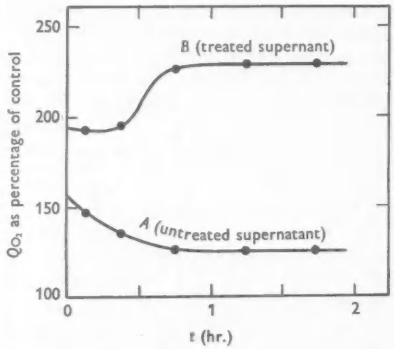

Fig. 8. Effect of supernatant fluid from treated cells on O_2-uptake of fresh cells. 'Treated' and 'untreated' supernatant obtained by centrifugation after 16 hr. incubation of the organisms in 0·15 M-K_2HPO_4 in presence of glucose ± *cis*-vaccenic acid (532·5 μM, $a:c = 1:20$); glucose added to 0·5 %. Fresh cells (2·4 mg. dry wt.) were suspended in fresh medium (0·15 M-K_2HPO_4 + 1 % glucose) and in the supernatant fluids. Q_{O_2} as percentage of control. *A*: in untreated supernatant; *B*: in treated supernatant.

only traces of the added acid. The same applies to dead (freeze-dried) bacteria. However, the supernatant fluid of treated cells affects the O_2 uptake of fresh cells in a manner shown in Fig. 8, where the data have been plotted as percentage of the Q_{O_2} of control cells against time, as in Fig. 4. There is a small (25 %) increase of the respiratory rate of cells when suspended in the supernatant fluid derived from untreated cells (Fig. 8, curve *A*) compared with cells suspended in a fresh medium. This is probably due to the diffusion of stimulating substances out of the bacteria during incubation. However, the increase of the oxidation rate of untreated cells in the supernatant from treated cells is considerably greater, amounting to 125 % (curve *B*). The rise of curve *B* for the first 45 min. seems to indicate that the optimum concentration inside the cells of a metabolite contained in the treated supernatant, and responsible for the rise in oxidation rate, is only gradually reached or that this substance required further metabolism by the bacteria.

The same experiment was carried out with the supernatant fluid of dead (freeze-dried) cells after incubation ± *cis*-vaccenic acid. Both supernatant fluids caused an increase of O_2 uptake by 80–90 % compared with cells in a fresh medium, which is due to substances extracted from the dead bacteria during incubation. There was, however, no difference between the oxidation rates obtained in either of the supernatant fluids. The effect shown in Fig. 8 therefore requires the activity of the living cell.

Aggregation. During incubation with *cis*-vaccenic acid ($a:c = 1:20$) marked aggregation of cells, visible macroscopically, occurred after about 3–4 hr. The aggregation can temporarily be broken by vigorous shaking of the suspension; this must be done immediately before the measurement when turbidity determinations are made, as the aggregates tend to reform quickly. A similar aggregation has been described by Saz (1949) for tubercle bacilli in presence of long-chain carbon compounds some of which inhibit the O_2 uptake of the bacilli. While Saz assumes that the inhibition of respiration of aggregating cells may be due to a decrease or change in the total free surface of the bacilli, no inhibition of the growth or respiratory rate of aggregated cells has been found in these experiments.

Effects of trans*-vaccenic acid.* A few experiments only have been carried out with *trans*-vaccenic acid in order to compare its activity with that of the *cis*-form. These concerned the effect on growth with the ratio $a:c = 1:20$, on O_2 uptake (Fig. 7) and on the rate of respiration after 16 hr. incubation with 1:25,000 acid ($a:c = 1:20$). In all three cases *trans*-vaccenic had about 40–50 % of the activity of the *cis*-form.

DISCUSSION

The 'double action' of fatty acids refers, in the first instance, to the very narrow margin between inhibiting and stimulating doses. The term might also be applied to the phenomenon, that the same concentration of acid, acting on a given amount of cells, produces at first an inhibition and later an increase of growth. Most workers have concentrated on one or other of the two aspects. Thus Williams & Fieger (1946), dealing with the growth-promoting effect of oleic acid on lactobacilli, state that the growth in presence of oleic acid exceeds that of the controls only after 72 hr., and have chosen the 72 hr. period for their acidimetric titration. They noted, however, that oleate, up to 48 hr., produced a marked inhibition both in presence and in absence of added biotin. Kodicek (1949), on the other hand, in studying the bacteriostatic effect of fatty acids, has mainly concentrated on the 48 hr. period, stating that even

with the most efficient inhibitor (linolenic acid) 'growth and acid production were depressed over a period of at least 72 hr.', according to the concentration of the acid. In his experiments the period of inhibition (48 hr.) seems also to have been followed by one of normal and, in the case of oleic acid, of increased growth. The two properties of unsaturated fatty acids, therefore, namely stimulation or inhibition either by varying concentrations of acid at a given time or by the same concentration of acid at different times, must be considered as closely related and, most probably, as having the same basis. Both effects have been observed in the present experiments, taking the rate of respiration as a criterion (see Fig. 4 at 180 min. and Fig. 5).

These experiments also show that inhibition of growth and of respiration are simultaneous. Below a certain concentration of acid the respiratory rates eventually regained or exceeded the initial level. When the respiratory rate returned to the initial value a lag period set in, except with the lowest concentration of acid, which was followed by normal growth. It seems, therefore, justifiable to conclude that inhibition of growth is due to a temporary blocking of the respiratory system, as would be expected to be the case, at least with a strictly aerobic organism, like *Bacillus subtilis*. A similar parallelism between bacteriostasis and inhibition of respiration has previously been described for linoleic acid acting on *Mycobacterium tuberculosis* (Bergström, Theorell & Davide, 1946). In their experiments the margin between non-effective and inhibitory doses of linoleic acid was very narrow and, with higher doses, bacteriostasis led, after about 18 hr., to the death of the cells. Two factors seem, therefore, to be involved in recovery from inhibition of growth and of respiration, especially with higher concentrations of acid, namely, the ability of the cell to survive protracted respiratory inhibition, and also the ability to overcome or reverse it. The former condition is probably fulfilled with the majority of aerobic bacteria, but may not be with *M. tuberculosis*, especially if toxic products of anaerobiosis accumulate.

The mechanism by which respiration is temporarily inhibited has not been established. However, a purely physico-chemical mechanism, acting by surface adsorption (for theory and literature see Kodicek, 1949) does not seem adequate for some of the observed effects, especially the reversibility of the respiratory inhibition, for this would necessitate, besides an active incorporation of fatty acids into the cell wall, a specific rearrangement with time of the adsorbed molecules coupled with a gradual binding by preformed inhibitors in the cell wall leading to innocuous complexes.

On the other hand, combination of the fatty acid with substances such as albumin, cholesterol and lecithin, which suppress the effect, is instantaneous, as has been shown in experiments on haemolysis by *cis*-vaccenic acid (Laser, 1949). The observed effects, therefore, especially the reversibility of respiratory inhibition, seem to point to reactions caused by acid which has penetrated into the cell. Slater (1949) has shown that detergents, especially *cis*-vaccenic acid, are non-specific inhibitors of the succinic-oxidase system, by affecting the mutual accessibility of the components of the system. It is therefore suggested that the inhibition of oxygen uptake is due to the effect of the acid on intracellular oxidizing catalysts, and the reversibility to the ability of the organisms to metabolize the acid which has entered.

It is not assumed that, under the experimental conditions, the whole of the acid added enters the cells because (1) the marked aggregation of the organisms on incubation with vaccenic acid indicates its persistent adsorption on the cell surface accompanied by physical changes in the cell membrane, (2) recovery of *cis*-vaccenic acid from the organisms immediately after its addition and after 16 hr. incubation ($a:c = 1:20$), as tested by the haemolytic activity of serial dilutions of the recovered acid (Laser, 1949), showed no significant difference between the two samples. Unless the metabolic product of the acid has the same haemolytic activity as the original acid, the amount which, it is assumed, penetrates the cell and is metabolized is only a small fraction ($< 1\%$) of the added amount, too small to be detected by difference in the haemolysis test. Nevertheless, since the ratio $a:c$ in these experiments was 1:20, and complete inhibition of the succinic oxidase system was shown by Slater to occur at a concentration of 1:4000, such an amount, though undetectable, might be effective.

It has been commented upon by several workers (Bergström *et al.* 1946; Pollock, 1949) that commonly occurring acids, like oleic acid, to which parasitic bacteria are normally exposed in their natural environment, produce a marked bacteriostatic effect *in vitro*. The experiments dealing with repeated addition of acid offer an explanation for this discrepancy, as growth and respiration were unaffected by a second addition of acids to cells which had recovered from the primary inhibition. Here too it is improbable that the effect is due solely to surface phenomena, such as the formation of a protective surface film around the cells making them inaccessible to a second amount of added acid, partly for the reasons already stated, but mainly because resistance to renewed exposure to acid persisted even after prolonged (16 hr.) incubation with and growth in presence of acid. By this time the original cell population had increased several fold, i.e. the ratio $a:c$ had changed from 1:20 to $< 1:100$. Even assuming that each new cell had a protective film of vaccenic acid molecules, it seems difficult to visualize

that it should protect cells from a further addition of acid leading to a ratio $a:c = 1:11{\cdot}5$ in view of the fact that initial addition of acid at ratios $a:c = 1:20$ or $1:100$ produced quantitatively a very marked difference.

It is suggested, therefore, that on first addition of acid a penetration gradient is established, and that, within limits, at each concentration of acid a fraction which varies with the concentration enters the cell; once this process has come to a steady state, thereby inhibiting the respiration, the entered acid is gradually metabolized, and the end product causes an increase in the oxygen uptake and, under suitable conditions, stimulates the growth rate. The resistance of the organisms to further addition of acid may be due to a process resembling the acquired drug resistance of bacteria. The processes may not, however, be entirely analogous, as in the present case the cells were grown throughout in presence of vaccenic acid and had not been subcultured in its absence after recovery from the initial growth inhibition. Resistance in these experiments, furthermore, also developed in washed cell suspensions, which is not the case in acquired drug resistance, except in the case of the acridines. It is hoped to examine this point in further experiments. However, the conditions with regard to the continuous presence of, and exposure to, the acid are similar to those obtaining in the body.

The greater efficiency of the *cis*- than of the *trans*-form of the acid has also been observed with other acids for either growth inhibition (Kodicek, 1949), or for growth promotion (Shull *et al.* 1949).

SUMMARY

1. *cis*-Vaccenic acid, within the range of the concentrations used, produces a concomitant inhibition of growth and respiration of *Bacillus subtilis*.

2. The respiratory inhibition is transient, its degree and duration being dependent on the ratio of acid to cells.

3. The organisms, after recovering from the inhibition of oxygen uptake, respire at a considerably increased rate and grow, in presence of yeast extract, at a normal or slightly increased rate.

4. Growth and respiration of acid-treated recovered cells are unaffected by a second addition of acid.

5. The supernatant fluid from treated recovered cells causes an increase in respiration of fresh cells.

6. It is concluded that physico-chemical surface phenomena cannot by themselves account for all the observed facts, which can be more readily explained on the basis of a penetration of acid into the cell, an inhibition of the oxidative system inside the cell, an ability of the organisms to metabolize penetrated acid, and the development in treated cells of a mechanism resembling that in acquired drug resistance.

7. *trans*-Vaccenic acid has about half the activity of the *cis*-form.

I am greatly indebted to Dr I. D. Morton for supplying me with the synthetic vaccenic acid.

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Tricalcium Phosphate as an Adsorbent in the Chromatography of Proteins

By S. M. SWINGLE* AND A. TISELIUS
The Institute of Biochemistry, University of Uppsala

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The recent spectacular success of the chromatographic method in solving many important problems in diverse fields of chemistry and biology has encouraged investigators in many laboratories to anticipate comparable success in the chromatography of proteins. However, in spite of the great interest in the problem and several encouraging preliminary experiments, a well developed method for general use has not yet appeared. The use of batch procedures involving consecutive adsorption and elution, particularly on aluminium hydroxide, bauxite, tricalcium phosphate and certain silicate minerals, is well known to enzyme chemists and some workers have also used chromatographic procedures (see especially Bamann & Myrbäck, 1941; Sumner & Somers, 1947 and Zechmeister, 1950).

On the basis of his observations on 'salting-out adsorption' on paper and silica gel, Tiselius (1948) suggested the possibility of protein chromatography using this method. Mitchel, Gordon & Haskins (1949) were able to separate considerable quantities of enzymes by salting-out adsorption on a paper 'chromatopile'. Franklin & Quastel (1949) have reported paper chromatography of proteins under conditions not involving salting out, although experiments by one of us (S.M.S.) indicate that such chromatograms may be artifacts resulting from the combined effects of the viscous resistance to flow offered by the protein spot and the irreversible adsorption of small quantities of protein in an immobile tail. Sober, Kegeles & Gutter (1949) performed a successful frontal analysis of egg white proteins using a cation-exchange resin.

The fact that chromatography of proteins has not yet had the brilliant success achieved, for example, with the amino-acids is probably in part due to the limitations imposed by having to work with systems that do not promote denaturation; this confines investigation to mild adsorbents and to aqueous media free of such detergents and organic solvents as would otherwise be chosen as promising eluting agents. It has been considered worth while in this Institute to extend the range of possible adsorbents by studying a variety of materials and to compare their behaviour critically. Shepard & Tiselius (1949) studied extensively the adsorption of several proteins on silica gel and conducted promising frontal analyses of mixtures, although attempts at elution or displacement were discouraging (private communication). The present paper reports similar studies with tricalcium phosphate, with reference to the effect of salt and of changes of pH on the elution analysis of phycoerythrin in phosphate buffers. Quantitative comparisons with some other adsorbents are also reported.

* Present address: Eli Lilly and Co., Indianapolis, Indiana, U.S.A.

MATERIALS AND METHODS

Preparation of adsorbents. Hydrated tricalcium phosphate was prepared by the slow addition of conc. H_3PO_4 to a chilled solution of a calcium complex of sucrose, according to a method developed by MacIntire, Palmer & Marshall (1945). To 450 g. pure sucrose in 2 l. of water were added 75 g. CaO prepared by calcining 150 g. of precipitated $CaCO_3$ at 1000° for 3 hr. The suspension was agitated periodically for several hours until most had dissolved, and was clarified. Conc. H_3PO_4 (about 18 ml.) was added dropwise with continuous stirring over the course of an hour to 800 ml. of the calcium-sucrose solution, chilled to about 5°, until the pH dropped to 9·5; after this the stirring was continued for 4 hr. The resulting precipitate of tricalcium phosphate was thoroughly washed in a large centrifuge with many portions of distilled water. No special precautions were taken to exclude CO_2. The product, a voluminous finely divided gelatinous material, was stored and used as a suspension in water (about 0·08 g./ml.).

Proteins. Phycoerythrin, the fluorescent red protein from the sea weed *Ceramium rubrum*, was prepared by extraction and fractional crystallization according to Kylin (1910). Bovine serum albumin, obtained through the courtesy of Dr K. O. Pedersen, had been prepared by $(NH_4)_2SO_4$ fractionation, and was reprecipitated before use.

Chromatographic technique. Perspex columns (62 mm. long and 8 mm. diam.) were packed with a suspension containing 1 part by weight of tricalcium phosphate (dry weight basis) and 5 parts Super-Cel filter aid (Eimer and Amend, New York). The Super-Cel was first freed of foreign matter and larger particles by allowing a suspension to settle for a few seconds and then decanting the remaining more finely divided material for use. The column was packed by forcing the mixture as a fairly heavy suspension into the column from the top reservoir under a pressure of 3 kg./sq.cm. The column was thus tightly packed in a few minutes, after which the top was gently smoothed and packed with a spatula. The application of the spatula tended to squeeze out water, making the top layer very moist and fluid, and it was

necessary to remove this moisture by intermittent gentle suction from below, taking care not to draw air into the column. The assembled column was washed with 1% (w/v) aqueous NaCl, and the sample of phycoerythrin (0·5 ml. of 0·2% (w/v) solution in saline) was pressed in and further washed with a few ml. saline. The eluting agent to be studied was then added to the reservoir and a few cm. Hg pressure applied as necessary to produce a flow rate of 2–3 ml./hr., corresponding to a linear velocity of the solution of about 50 mm./hr. After elution commenced, the volume of effluent solvent and the corresponding travel of the red band of protein were measured. The dead volume of the column was determined by frontal analysis of a solution of glucose (5%, w/v) under the assumption that the glucose was not adsorbed.

Adsorption isotherms. The adsorption of serum albumin on various materials was studied in batch experiments in which the solvent, adsorbent and protein with a total volume of 12 ml. were mixed, in the order given, in test tubes which were then tightly stoppered with the exclusion of bubbles and rotated end over end about 10 times/min. for several hours. The adsorbent was then removed by centrifugation and filtration or by two consecutive centrifugations, and the supernatant was analysed for protein by ultraviolet absorption at 276 mμ. The measured concentration was corrected by the use of blanks from which the protein was omitted, and the result subtracted from controls (having only protein and solvent, but no adsorbent) to give the amount of protein adsorbed.

RESULTS

The elution analysis of phycoerythrin

The effects of variations in pH, sodium chloride concentration and phosphate buffer concentration are presented in Table 1, which gives the velocity of the protein as a fraction of that of the solution (R_F), and these data are graphically illustrated in Fig. 1. Phosphate buffer is an effective eluting agent for this system so that in concentrations above 0·04 M-phosphate in 1% (w/v) saline there is rapid elution of the protein, while in the absence of phosphate there is no discernible motion of the band. The band moved with half the velocity of the solvent in 0·01 M-phosphate in 1% saline. The effect of salt was more startling and more complicated. In the absence of salt there was very little retention of the protein,

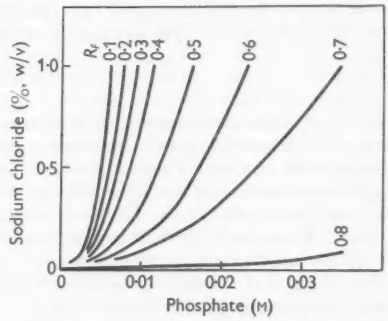

Fig. 1. Iso-elution curves for phycoerythrin on tricalcium phosphate. The curves represent interpolated values for the relative motion of protein and solvent (R_F) as a function of salt concentration and phosphate concentration at pH 7·8 (see Table 1).

Table 1. *The elution analysis of phycoerythrin in various solutions*

Sodium phosphate buffer concn. (M)	pH	NaCl concn. (%, w/v)	Elution velocity (R_F)	
			Protein	Phosphate
0·00	—	0	0·90	—
0·0025	7·8	0·1	0·10	—
0·0025	7·8	0·05	0·10	—
0·0025	7·8	0·002	0·83	—
0·006	5·2	1·0	0·10	—
0·005	7·8	0·5	0·10	0·43
0·005	7·8	0·25	0·26	—
0·005	7·8	0	0·78	—
0·007	6·7	1·0	0·34	—
0·008	7·8	0·5	0·37	—
0·01	7·8	3·0	0·30	—
0·01	7·8	1·0	0·38	0·60
0·01	7·8	0·25	0·56	—
0·0125	7·8	1·0	0·43	—
0·0125	5·2	1·0	0·52	—
0·0125	7·8	0	0·83	—
0·0125	5·2	0	0·78	—
0·0175	6·7	1·0	0·53	—
0·025	7·8	5·0	0·48	—
0·025	7·8	1·0	0·63	0·65
0·025	5·2	1·0	0·59	—
0·025	7·8	0	0·83	0·85
0·035	6·7	1·0	0·71	—
*	8·0	1·0	0·00	—

* 0·02 M-sodium diethylbarbiturate buffer.

even at low phosphate levels. Experiments in distilled water were not always reproducible, presumably because of the failure to control the pH, but in some cases R_F 0·9 was obtained, whereas retention was very strong when even traces of salt were present. More reproducible results were obtained in 0·0025M-phosphate, where it was found that the addition of as little as 0·05 % sodium chloride would cut the R_F from 0·85 to as little as 0·1.

In Fig. 1 the relationship between the effects of salt and phosphate is illustrated by plotting lines of equal protein elution velocity, expressed as R_F on a graph of salt concentration against phosphate concentration. The graph is drawn only for concentrations below 1 % saline, where most of the effect occurs. Somewhat surprisingly, there was no effect of pH variations between 5·2 and 7·8 within the experimental errors. Even pH 8·0 sodium diethylbarbiturate buffer would not give elution in the absence of phosphate ions. The possibility that pH effects exist below 0·01M-phosphate was not studied.

The phycoerythrin band was usually very regular, indicating that the packing was uniform, although a pronounced upward convexity of the front was apparent on the extruded and sectioned adsorbent column. Under conditions where the retention was slight ($R_F > 0·6$), the band was very narrow and well defined, but invariably some material of a slightly darker red colour was left at the top of the column. In addition, there was a faint trace of colour uniformly distributed over the column, presumably as a result of slight irreversible tailing. With higher retention there was a progressively more diffuse tail behind the sharp front and more material left near the starting point in a diffuse band so that chromatograms with R_F less than 0·3 appeared almost continuously coloured with phycoerythrin up to the sharp leading edge, this edge being used in the measurements. When stronger phosphate was sent through such a chromatogram it would pick up almost all of the trailing material as a second sharp band which would eventually catch up and merge with the leading band. In a series of exploratory experiments, a mixture of phycoerythrin and the blue phycocyan were readily separated, with the red band leading.

In interpreting the results and planning new experiments it is important to know the position of the phosphate front relative to the protein in order to decide whether the phenomenon is essentially displacement or elution. This was checked at four typical concentrations, using ammonium molybdate as a test reagent for the appearance of the phosphate front in the effluent, and the corresponding values of the velocity of the front relative to the solvent are reported in Table 1. In 0·01M-phosphate in 1 % saline, where the elution of the protein proceeded at an intermediate rate and showed considerable tailing for several mm. behind the front, the phosphate front was well ahead of the phycoerythrin, indicating that the phenomenon is true elution. On the other hand, under conditions where the protein moves rapidly in a narrow band, the phenomenon appears to be essentially displacement, with the protein band lying within the concentration gradient of the phosphate front, which may account for its extreme sharpness. This was checked at a phosphate concentration of 0·025M in 1 % saline, in which case the presence of phosphate in the effluent was first detected three drops before the appearance of the phycoerythrin. The other values reported for the mobility of the phosphate front, as measured in the absence of protein, similarly indicate that the phenomenon may be either elution or displacement, depending on whether the protein retention is small or great.

Preliminary experiments with serum albumin gave elution in a sharp band. However, the serum albumin was more strongly retained and required 0·03M-phosphate for moderately fast elution. In an attempted chromatographic analysis of partially purified D-amino-acid oxidase, done in collaboration with Dr W. Frisell, the elution was accomplished with successive portions of phosphate buffer at progressively increasing concentrations. The activity was eluted in two sharp bands, one carried along in 0·005M- and the second in 0·02M-phosphate. The suggestion is made that the second band might have been material left as a tail behind the first band and subsequently picked up by the more concentrated phosphate. The total recovery of enzyme activity was 61 %. When the experiment was repeated using only 0·025M-phosphate, the recovery was again 61 %, with the activity appearing in a single band.

Comparison with other adsorbents

A number of isotherms were determined for the adsorption of bovine serum albumin on various materials. These isotherms were curved near the origin and tended to level off to a saturation value when the protein concentration in the supernatant reached about 0·05 %. Since these studies are incomplete, only the approximate adsorption value at this supernatant concentration will be reported here for purposes of comparison. Paper (Munktell 3-Grycksbos Pappersfabriks AB, Grycksbo, Sweden) at pH 5·0 or 5·5 gave salting-out adsorption amounting to 0·5 mg. protein/g. paper in 1·5M-ammonium sulphate, 1·5 mg./g. in 2·0M, and 4·0 mg./g. in 2·5M. A Brockmann-type aluminium oxide buffered at pH 5·0 with 0·1M-sodium acetate buffer gave an optimum adsorption in 0·5M-ammonium sulphate of about 10 mg./g., and about half this adsorption in 1·0M-ammonium sulphate or in its complete ab-

sence. A sample of silica gel prepared by Dr R. L. M. Synge for chromatographic use gave an adsorption of 110 mg./g. at pH 5·0 without the addition of ammonium sulphate, and a commercial anhydrous silicic acid gave comparable results.

The hydrated tricalcium phosphate used in the chromatographic experiments reported here was studied only in the absence of ammonium sulphate, and adsorbed serum albumin from a 0·005 M-sodium acetate buffer (pH 5·0) to the extent of about 310 mg./g., calculated on the dry weight basis. Addition of 0·05 M-sodium phosphate buffer (pH 6·3) reduced the adsorption to about one-fifth, and gave a nearly linear isotherm as far as the curve was studied (up to 0·1 % (w/v) of protein in the supernatant solution). The adsorption was reduced to 130 mg./g. by 0·03 M-phosphate buffer at pH 6·3. A sample of tricalcium phosphate precipitated from trisodium phosphate by the addition of calcium chloride had similar adsorption characteristics, but a commercial anhydrous material showed no adsorption at all. Visually estimated experiments with the adsorption and subsequent phosphate elution of phycoerythrin on gamma aluminium hydroxide (Willstätter) indicated that its behaviour was similar to that of tricalcium phosphate.

DISCUSSION

On the basis of these preliminary experiments, precipitated tricalcium phosphate seems to be a promising material for protein chromatography, being readily prepared in a reproducible form with desirable physical characteristics, and showing reversible adsorption for many proteins. Certainly it is worthy of further investigation. The effectiveness of phosphate buffers as eluting agents probably reflects the tendency of the calcium phosphate crystals to attract ions of the same kind from the solution, thereby decreasing their affinity for protein, and it is to be noted that the phosphate solutions show considerable retention on the column. The more surprising effect of small salt concentrations in promoting the adsorption of phycoerythrin cannot be so readily explained, and a theoretical discussion seems out of order until more data are available. It is to be noted that salt also considerably increases the retention of the phosphate buffer, but that the effect on the protein predominates. The absence of a marked pH effect is in sharp contrast with the behaviour of silica gel.

A possible difficulty with the system is the tendency for the protein to tail behind the front when the elution is slow. This tailing, which seems to occur particularly when the phosphate front is ahead of the protein, is probably a consequence of the curving of the adsorption isotherm, and it is difficult to predict whether it will prove an obstacle in the analysis of mixtures. The elimination of tailing in higher phosphate concentrations appears to be the result of almost complete displacement of the protein which is then carried along in the phosphate front, and suggests the possible advantage of eluting with a progressively increasing phosphate concentration. The material left at the starting point is probably a mixture of impurities and denatured protein already present in the sample used. Whether the process of adsorption analysis itself causes any denaturation is a question which should be thoroughly investigated. The experiments with D-amino-acid oxidase had that point as one objective (as any denaturation should cause a loss in enzymic activity) and established that the tendency toward denaturation was certainly not great. However, considerably more work will be necessary to prove that the danger of denaturation can be ignored.

SUMMARY

1. Hydrated tricalcium phosphate as an adsorbent for proteins has been investigated with respect to the effects of phosphate buffer, pH and sodium chloride on the chromatography of phycoerythrin.

2. Phosphate buffer is an effective displacing agent. In low concentrations it gives elution analysis of phycoerythrin with considerable tailing, and in higher concentrations it gives displacement analysis in sharp bands. Variations in pH have little effect.

3. Sodium chloride in low concentrations promotes to a great extent the adsorption of phycoerythrin, and to a lesser extent the adsorption of phosphate buffer.

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The Metabolism of Nitrate and Nitrite in the Sheep

1. THE REDUCTION OF NITRATE IN THE RUMEN OF THE SHEEP

By D. LEWIS*
Biochemical Laboratory University of Cambridge

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A disease of cattle following the ingestion of young oat hay or straw, referred to as 'oat hay poisoning', was reported in the U.S.A. by Newsom, Stout, Thorp, Barber & Groth (1937). Subsequently, Bradley, Eppson & Beath (1939) were able to produce the disease in the laboratory by feeding a steer on the affected hay. Analysis of a number of samples of toxic hay revealed that in all cases nitrate was present in abnormally high concentrations—3·2–7·2% of the dry matter. Bradley, Eppson & Beath (1940) showed that the ingestion of potassium nitrate produced the same symptoms, and simultaneously observed a severe methaemoglobinaemia. It was found that 1 g. potassium nitrate/kg. body weight was a lethal dose, and in a few hours resulted in the conversion of 70–80% of the haemoglobin into methaemoglobin. Sheep were affected in the same way. Since nitrite is known to convert haemoglobin into methaemoglobin, it was concluded that in the alimentary tract of the ruminant nitrate was reduced to nitrite and that the latter gave rise to the methaemoglobinaemia. A similar disorder, 'tribulosis', had been reported many years previously in South Africa (Rimington & Quin, 1933; quoted by Russell, 1944). Symptoms resembling those of 'tribulosis' were produced by dosing sheep with the expressed juice of species of *Tribulus* or with potassium nitrate itself. While the present work was in progress the South African group reported that incubation of untreated rumen contents *in vitro* with nitrate resulted in the formation of nitrite (Sapiro, Hoflund, Clark & Quin, 1949).

The present investigations were designed to provide information concerning (*a*) the reduction of nitrate to nitrite *in vivo* in the rumen of the sheep, and the further reduction of nitrite to ammonia; (*b*) the changes, if any, in the ammonia content of the rumen fluid subsequent to dosing with nitrate; (*c*) the degree of methaemoglobinaemia produced after different amounts of nitrate or nitrite are placed in the rumen, and after the intravenous injection of known amounts of nitrite.

METHODS

The experimental sheep were Oxford Down × Halfbred 3-year-old wethers, fitted with a permanent rumen fistula as described by Phillipson & Innes (1939). They were kept in metabolism cages and fed 3·5 lb. (1·6 kg.) good meadow hay daily, with water *ad lib.* The weights of the sheep remained constant, ±1 kg., throughout the period of study. Urine samples, free from faeces, were collected under toluene. Blood samples were taken from the jugular vein, and clotting was prevented by sodium oxalate.

Samples of rumen contents were withdrawn through the fistula and the material so obtained filtered through muslin. The filtrate is termed rumen liquor (RL). All analyses of RL were carried out on a clear filtrate obtained by the following procedure: 2 ml. saturated lead acetate were added to 10 ml. of RL followed by 0·5 g. decolorizing char-

Table 1. *Recovery of nitrate added to rumen liquor, urine and water*

Material analysed	Nitrate added (mg. NO_3-N/100 ml.)	Colorimetric method		Conway method		Fe/H_2SO_4 reduction	
		Found (mg./100 ml.)	Recovery (%)	Found (mg./100 ml.)	Recovery (%)	Found (mg./100 ml.)	Recovery (%)
Rumen liquor	0	0·36	—	0·33	—	0·32	—
	10	9·7	93	9·8	95	9·3	90
	20	19·4	95	19·8	97	18·5	91
	30	29·2	96	29·4	97	28·5	94
Urine	0	0·45	—	0·24	—	0·20	—
	20	22·9	112	19·6	97	18·6	92
	40	34·7	85	38·2	95	37·4	93
Water	0	—	—	—	—	—	—
	5	4·95	99	4·90	98	5·05	101
	10	9·85	99	10·00	100	9·73	97

* Present Address: Department of Bacteriology, University of Sheffield (seconded from the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge).

coal; after shaking, 3 ml. saturated Na_2SO_4 were added to precipitate the excess lead, and the mixture filtered through a Whatman no. 1 filter paper. This procedure yielded a colourless clear filtrate. Urine samples were clarified in the same way, but the solutions were always very faintly coloured. All doses of $NaNO_3$ and $NaNO_2$ were administered to the sheep as 10% (w/v) aqueous solutions, unless otherwise stated.

Nitrite plus nitrate in RL filtrates were determined by the colorimetric phenoldisulphonic acid method (Association of Official Agricultural Chemists, 1947) using a Spekker photoelectric absorptiometer with a spectral violet Ilford filter no. 601 (maximum transmission 430 mμ.). Urine filtrates were unsuitable for the colorimetric method and were therefore analysed by the following volumetric methods. Preformed NH_3 and total nitrite and nitrate were determined by NH_3 estimations before and after reduction either by Devarda's alloy (Conway, 1947) or by powdered Fe and 8% (w/v) H_2SO_4 (Lees & Quastel, 1946). Table 1 shows that the three methods gave comparable results, with recoveries of nitrate added to normal RL consistently above 90%.

Nitrite was estimated by the Griess-Ilosvay method (Association of Official Agricultural Chemists, 1947) using a King photoelectric colorimeter with a green filter (maximum transmission 520 mμ.). Recoveries of nitrite added to RL or urine were within the limits of 91–99% and duplicates agreed ± 3%.

Methaemoglobin and total haemoglobin were determined by the method of Evelyn & Malloy (1938) using the Beckman spectrophotometer.

RESULTS

Metabolism of nitrate in the rumen

The nitrate and nitrite concentrations in the rumen of the untreated animal are uniformly low throughout a 24 hr. period. However, the ammonia concentration rises after feeding (McDonald, 1948), a fact which complicates the interpretation of the ammonia values if the animal is dosed with nitrate at the time of feeding.

McDonald (1949), however, has shown that the ammonia content of rumen liquor is relatively constant during the last 8 hr. before feeding. This observation suggested an experimental procedure which would permit an assessment of the ammonia formation from nitrate. The animal was fed at 6 p.m., and samples of RL were collected from 8 a.m. the following morning. This experiment served as a control; it may be seen from Fig. 1 that the ammonia level remained constant, thus confirming McDonald's observation. The procedure was repeated save that 12 g. sodium nitrate were introduced into the rumen 16 hr. after feeding.

The results (Fig. 1) show that there was a rapid fall in the nitrate, coincident with the production of nitrite which, however, disappeared during the succeeding 5 hr. In the control experiment the ammonia concentration remained constant throughout, whilst after the addition of nitrate there was a definite rise lasting 5–7 hr. This may be due to a stimulation of ammonia production by the nitrate or a conversion of the nitrate to ammonia. The latter is the more probable, since only a small proportion of the nitrate is accounted for in the urine or blood, and, as will be shown later, it cannot all be absorbed as nitrite, for such a quantity would be far in excess of the lethal dose. In this experiment excretion in the urine accounted for less than 8% of the nitrate introduced into the rumen: the nitrite content was very low, and the ammonia values variable.

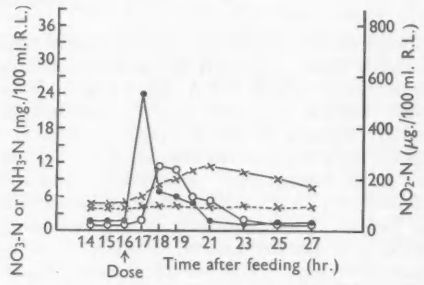

Fig. 1. Nitrate, nitrite and ammonia in RL after placing 12 g. $NaNO_3$ in the rumen, 16 hr. after feeding. ×- - -×, control NH_3-N (mg./100 ml. RL); control NO_3-N and NO_2-N negligible; ×—×, experimental NH_3-N (mg./100 ml. RL); ●—●, experimental NO_3-N (mg./100 ml. RL); ○—○, experimental NO_2-N (μg./100 ml. RL).

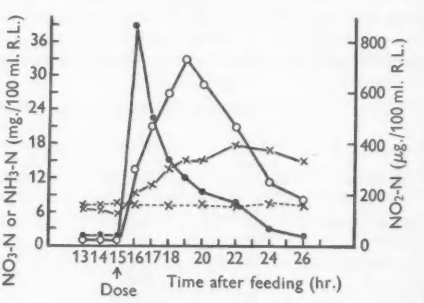

Fig. 2. Nitrate, nitrite and ammonia in RL after introducing 25 g. $NaNO_3$ into the rumen, 15 hr. after feeding. ×- - -×, control NH_3-N (mg./100 ml. RL); control NO_3-N and NO_2-N negligible; ×—×, experimental NH_3-N (mg./100 ml. RL); ●—●, experimental NO_3-N (mg./100 ml. RL); ○—○, experimental NO_2-N (μg./100 ml. RL).

The changes in the nitrate, nitrite and ammonia of the RL, following a dose of sodium nitrate sufficient to produce a severe methaemoglobinaemia, were next investigated, 25 g. sodium nitrate being given to the sheep 15 hr. after feeding. A control experiment was carried out in which no nitrate was administered. The results are presented in Fig. 2. The methaemoglobin content of the blood had

reached its maximum 7 hr. after dosing and amounted to 63 % of the total haemoglobin.

The results show the same general tendencies as in Fig. 1, when half this quantity of sodium nitrate was administered, but the increase in nitrite was much more marked. The nitrate, nitrite and ammonia concentrations in the RL were constant in the control experiment. The nitrate concentration 1 hr. after dosing was 39 mg. NO_3-N/100 ml. RL (25 g. sodium nitrate in 6 l. RL is equivalent to 68·6 mg. NO_3-N/100 ml. RL). Whilst the nitrate concentration fell rapidly there was still an appreciable amount present 6 hr. after dosing. The nitrite concentration rose steadily for 4 hr. and fell uniformly almost to normal during the next 6 hr., but there was still a considerable amount of nitrite (460 μg. NO_2-N/100 ml. RL) present in the RL 7 hr. after dosing, at which time the methaemoglobin content of the blood was at its maximum. There was a pronounced rise in the ammonia concentration, which presumably accounted for a considerable proportion of the nitrate disappearing at the later stages. Only a small proportion (4 %) of the nitrate was lost in the urine.

Nitrate and methaemoglobin formation

A series of experiments was carried out in which the methaemoglobin content of the blood was determined after increasing quantities of sodium nitrate (0–25 g.) had been placed in the rumen. A different quantity was administered daily, and control experiments, in which no nitrate was administered, were carried out at the beginning and at the end of the series. The results of these control experiments showed excellent agreement. After dosing, samples of blood were collected at intervals over the succeeding 8 hr. period and analysed for total haemoglobin and methaemoglobin. The haemoglobin contents of all samples lay within the range of 11–13 % (w/v) of the blood. The results are given in Fig. 3.

As the quantity of sodium nitrate administered was increased from 12 to 22·5 g. the extent of the methaemoglobin production gradually became greater, and the time between administration and the maximum value attained became longer. When the dose was increased to 25 g. sodium nitrate there was a sharp rise in the maximum methaemoglobin. This may be due to the fact that above a certain concentration of nitrate the rate of reduction of nitrite to ammonia becomes limiting, and in consequence nitrite accumulates in the rumen. This nitrite passes into the blood in increasing concentration and gives rise to a severe methaemoglobinaemia.

Nitrite and methaemoglobin formation

A further series of experiments was carried out in which sodium nitrite was placed in the rumen. The quantity administered was increased daily as with nitrate. Samples of blood were collected and methaemoglobin estimated (Fig. 4). The administration of less than 5 g. sodium nitrite (5 % (w/v) solution) had but little effect on the methaemoglobin content of the blood. There was a significant methaemoglobinaemia when 7 or 9 g. sodium nitrite were placed in the rumen, with a further increase in the maximum methaemoglobinaemia attained when the dose was increased to 10 g. sodium nitrite. The length of time between the administration and the peak values again became greater as the dose was raised.

The results of the analysis of samples of RL taken at intervals after 10 g. sodium nitrite were placed in the rumen are presented in Table 2, and the results obtained following an equivalent dose of sodium nitrate, are included for comparison. The nitrite concentration remained above 500 μg. NO_2-N/100 ml.

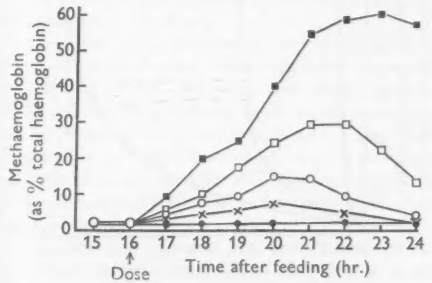

Fig. 3. Methaemoglobin production when various amounts of $NaNO_3$ are placed in the rumen, 16 hr. after the sheep was fed. ●—●, Exp. 3, 0 g. $NaNO_3$; ×—×, Exp. 4, 12 g. $NaNO_3$; ○—○, Exp. 5, 17·5 g. $NaNO_3$; □—□, Exp. 6, 22·5 g. $NaNO_3$; ■—■, Exp. 7, 25 g. $NaNO_3$.

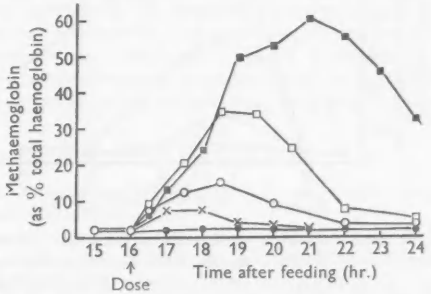

Fig. 4. Methaemoglobin production when various amounts of $NaNO_2$ are placed in the rumen, 16 hr. after the sheep was fed. ●—●, Exp. 10, 1 g. $NaNO_2$; ×—×, Exp. 11, 5 g. $NaNO_2$; ○—○, Exp. 12, 7 g. $NaNO_2$; □—□, Exp. 13, 9 g. $NaNO_2$; ■—■, Exp. 14, 10 g. $NaNO_2$.

Table 2. NH_3-N *and* NO_2-N *in rumen liquor, and methaemoglobin in blood, after placing* 10 *g.* $NaNO_2$ *or* 12 *g.* $NaNO_3$ *in the rumen*

(The dose was given 16 hr. after feeding.)

Time after feeding (hr.)	Exp. 16, 10 g. $NaNO_2$ in rumen			Exp. 4, 12 g. $NaNO_3$ in rumen		
	NH_3-N (mg./100 ml. RL)	NO_2-N (μg./100 ml. RL)	Met. Hb as % total Hb	NH_3-N (mg./100 ml. RL)	NO_2-N (μg./100 ml. RL)	Met. Hb as % total Hb
15	4·5	12·5	2·0	4·0	10	1·5
16	4·6	10·0	2·3	4·0	11	1·7
17	5·4	17,500	13	4·9	20	2·5
18	7·0	8,100	24	9·8	248	4·0
19	9·4	2,750	49	10·8	232	5·5
20	9·9	1,250	53	10·9	122	8·0
21	9·3	570	61	11·1	100	—
23	8·1	120	46	10·2	26	4·9
25	5·7	30	32	9·0	18	—
27	4·9	15	—	8·0	16	—

RL for 5 hr. after the administration of the sodium nitrite while the concentration after administration of 12 g. sodium nitrate was negligible by comparison. However, the changes in ammonia concentrations were very similar, supporting the concept that nitrate is reduced in the rumen to ammonia, with nitrite as an intermediate. Nitrite and nitrate excretion was negligible.

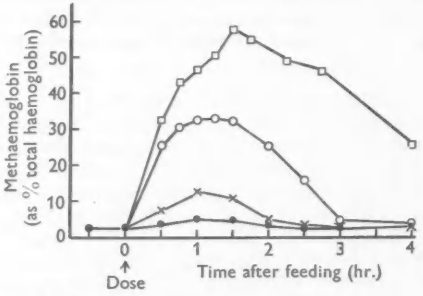

Fig. 5. Methaemoglobin production on the intravenous injection of $NaNO_2$. ●—●, Exp. 17, 0·5 g. $NaNO_2$; ×—×, Exp. 18, 1 g. $NaNO_2$; ○—○, Exp. 19, 1·5 g. $NaNO_2$; □—□, Exp. 20, 2 g. $NaNO_2$.

For comparison a series of experiments was carried out in which sodium nitrate was injected into the jugular vein in order to determine the amount of methaemoglobin produced by the interaction of known amounts of nitrite and the haemoglobin of the circulating blood. Samples of blood and urine were collected at intervals. The nitrite excreted in the urine was again negligible and in no case accounted for more than 1–2 % of the sodium nitrite given. A conversion of 60 % of the blood haemoglobin into methaemoglobin resulted when 2 g. sodium nitrite were injected (Fig. 5). The rise of methaemoglobin to its maximum was rapid and there was also a sharp increase in the methaemoglobin maximum as the dose was increased (Fig. 6). This is to be expected since in this experiment the nitrite and haemoglobin were brought into immediate contact, and the rate of methaemoglobin formation was independent of the rates of reduction processes or of passage through the rumen wall.

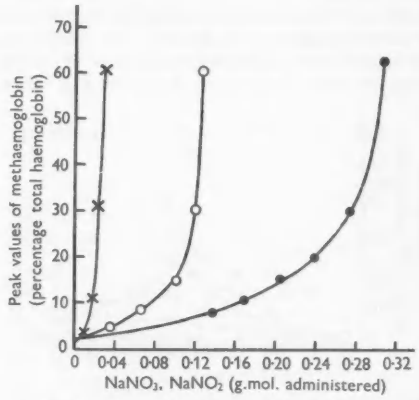

Fig. 6. Peak values of methaemoglobin plotted against dosage. ●—●, nitrate placed in the rumen; ○—○, nitrite placed in the rumen; ×—×, nitrite injected intravenously.

The peak methaemoglobin values produced on introducing various amounts of nitrate or nitrite into the rumen are compared in Fig. 6 with those resulting from the intravenous injection of nitrite. When less than 0·25 mol. nitrate or 0·11 mol. nitrite was added to the rumen, there was an approximately linear relationship between the amount administered and the extent of the methaemoglobinaemia. Greater quantities resulted in a disproportionately large formation of methaemo-

globin. This suggests that above a certain concentration of nitrite the rate of reduction of nitrite to ammonia is limited, and not as rapid as the reduction of nitrate to nitrite. In both cases nitrite would thus accumulate in the rumen, and presumably pass into the blood stream.

DISCUSSION

It is evident that nitrate is reduced to ammonia by the micro-organisms in the rumen of the sheep, and that nitrite is an intermediate in this reaction. The nitrite accumulates under certain conditions, and may lead to a production of methaemoglobin in the blood. A dose of 25 g. sodium nitrate or 10 g. sodium nitrite introduced into the rumen of the sheep (60 kg.) produces the same degree of methaemoglobinaemia (60 % of the total haemoglobin), as 2 g. sodium nitrite injected intravenously. The amount of nitrite injected intravenously may be expressed quantitatively in relation to the haemoglobin converted into methaemoglobin. The live weight of the sheep was 60 kg. and the blood haemoglobin level was 12 % (w/v). It is assumed that the sheep has a blood volume equivalent to 10 % of the live weight and that the iron content of the haemoglobin is 0·34 %. Knowing that 2 g. sodium nitrite administered intravenously converted 60 % of the haemoglobin to methaemoglobin it can be shown that approximately 4 mol. sodium nitrite are required to produce 1 mol. methaemoglobin. These figures are relevant to *in vivo* conditions and to the particular sheep employed.

The fact that 10 g. sodium nitrite introduced into the rumen has a similar effect suggested that only 20 % of the nitrite passed to the blood stream. Sodium nitrate (25 g.) placed in the rumen results in a similar degree of methaemoglobinaemia, but there are probably so many factors governing the methaemoglobin production in this instance that it is only possible to say that this is effectively equivalent to 10 g. sodium nitrite.

The bulk of the nitrate placed in the rumen is undoubtedly reduced to ammonia, and nitrite is an intermediate in this reaction. This nitrite can pass, to a greater or less extent, into the blood stream, where it leads to the conversion of part of the haemoglobin to methaemoglobin. Increasing the dose of nitrate to 22·5 g. sodium nitrate or more, led to a marked accumulation of nitrite in the rumen and a sharp increase in the extent of methaemoglobinaemia. It is concluded that at this level the rate of reduction of nitrite to ammonia becomes a limiting factor in the removal of nitrite, whereas further added nitrate is still reduced to nitrite. In the same way when nitrite is introduced into the rumen the reduction to ammonia becomes limited above a certain concentration of nitrite. In both cases, following doses of nitrate or nitrite greater than certain critical amounts the sheep will suffer from a methaemoglobinaemia that rapidly becomes more severe with larger doses, as was found experimentally (Fig. 6).

It is possible to correlate the peak methaemoglobin values with the changes in the nitrate, nitrite and ammonia in the rumen liquor after administration of nitrate. When 12 g. sodium nitrate was administered to the sheep (Fig. 1) the nitrate concentration fell off rapidly at first and then more slowly, while the ammonia values rose steadily for 5 hr. Nitrite was present in appreciable amount only for a short period during the initial rapid fall in the quantity of the nitrate present. It is possible that most of the ammonia, produced initially, enters the normal synthetic mechanisms in the rumen. The ammonia produced later would possibly be less utilized in this way owing to saturation of this mechanism and the rise in the concentration would be greater in proportion to the nitrate disappearing, on the whole fitting in with the observed steady rise in ammonia over several hours. This situation is still more obvious when 25 g. sodium nitrate were given to the sheep. Since the amount of nitrate present was still appreciable 5–6 hr. after administration the ammonia values increased over a longer period and to a higher level. The quantity of nitrite present in this case was greater, and persisted in the rumen for several hours. The extent of the methaemoglobinaemia increased to a peak of 60 % of the total haemoglobin at 7 hr. after dosing with nitrate.

SUMMARY

1. Nitrate introduced into the rumen of the sheep is reduced to ammonia.

2. Nitrite is an intermediate in this reaction and may accumulate under certain conditions and lead to a partial conversion of blood haemoglobin to methaemoglobin.

3. Sodium nitrate (25 g.) or sodium nitrite (10 g.) placed in the rumen, or 2 g. sodium nitrite injected intravenously, result in a methaemoglobinaemia corresponding to 60 % conversion of the total haemoglobin.

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The Use of Radioactive Isotopes in Immunological Investigations

5. QUANTITATIVE ASPECTS OF THE ANTIBODY-ANTIGEN REACTION

BY T. E. BANKS, G. E. FRANCIS, W. MULLIGAN AND A. WORMALL

Departments of Biochemistry and Physics, the Medical College of St Bartholomew's Hospital, London

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The reaction between antigen and antibody which results in precipitation has been studied quantitatively by Heidelberger, Marrack, Haurowitz, Kabat, Hooker and Boyd, and many other investigators (see reviews by Heidelberger, 1939, 1946; Marrack, 1938; Boyd, 1947; Kabat & Mayer, 1948; Wormall, 1948). These investigations have been based mainly on the determination of small amounts of nitrogen in the precipitates. The amount of antibody can be readily calculated from these determinations if the antigen contains little or no nitrogen (e.g. polysaccharides) or alternatively, the amounts of both antigen and antibody can be calculated, if the antigen contains some special group or element (e.g. dyestuff, iron, copper, etc.) which can be determined separately. Few natural proteins, however, contain a suitable label, and the introduction of foreign groups, leading to the production of unnatural proteins or protein complexes, is frequently undesirable. When unlabelled protein antigens are used, the analysis of the specific precipitates for both antigen and antibody is complicated, and may sometimes be impossible.

We have used antigens and antibodies labelled with radioactive isotopes to study the precipitin test and certain other serological reactions. At first our choice of antigen was limited to biologically prepared labelled proteins such as lipovitellin and vitellin (Francis & Wormall, 1948), and chemically altered proteins such as phosphorylated or mustard gas sulphone-treated proteins containing appreciable quantities of introduced phosphorus or sulphur (Boursnell, Dewey, Francis & Wormall, 1947; Banks, Boursnell, Dewey, Francis, Tupper & Wormall, 1948). With an improved supply of certain carrier-free radio-isotopes it later became possible, however, to use such small amounts of the element that the protein molecule could be adequately labelled without significant change in the properties of the protein other than the acquirement of radioactivity. The experiments described here were carried out in order (*a*) to obtain information about the best methods for labelling protein antigens and antibodies with radio-isotopes for serological investigations, and (*b*) to use these labelled compounds for quantitative studies on certain precipitin reactions. In most of these experiments chemically altered protein antigens have been used, e.g. those labelled with 8–12% radio-iodine; but where antibodies have been labelled with ^{131}I this has been effected with small amounts of radio-iodine of high specific activity. Preliminary notes of a few of these investigations have been published elsewhere (Boursnell *et al.* 1947; Banks, Francis, Mulligan & Wormall, 1949, 1950).

EXPERIMENTAL

Methods

Precipitin reactions. Since we usually varied the technique of these tests to suit the particular experiment undertaken, only the general details will be given here; exact details, where they are required, are given in the appropriate tables or parts of the text. The antigen and antibody solutions, plus

0·9% NaCl solution to bring to a standard volume, were mixed in tapered 15 ml. centrifuge tubes, and the mixtures kept at 37° for 3 hr. (or other specified time) and then overnight or for 2–4 days in the refrigerator (0–4°). The centrifuged precipitate, usually after it had been washed twice with small volumes of cold 0·9% NaCl, was dissolved in a small volume of dilute (e.g. 0·5 N) NaOH, and the solution diluted sufficiently to permit samples to be taken for all the radioactivity and chemical determinations. Since lipovitellin is almost insoluble in 0·9% NaCl solution, all precipitin tests with this antigen were carried out with mixtures containing about 10% (w/v) NaCl.

The washing of the antigen-antibody precipitates with 0·9% NaCl, to remove 'non-specific' proteins, was carried out in most of these experiments, for it has been shown that the amount of N in the specific precipitate is not thereby altered (Heidelberger & Kendall, 1932). The time required for complete precipitation of antigen-antibody complexes varies according to the system used and the experimental conditions, but it is generally agreed that with strong rabbit antisera precipitation is essentially complete in 1–2 hr. at 37° (see Kabat & Mayer, 1948, p. 36).

Total nitrogen determinations. These were made by the micro-Kjeldahl method, with the NH_3 distilled, in the apparatus described by Markham (1942), into a boric acid solution containing a methyl red-methylene blue indicator. The final titration was made with N/70-HCl, using an 'Agla' Micrometer syringe (Burroughs Wellcome and Co.) and with a stream of N_2 bubbling through the boric acid mixture. The method is generally similar to that described by Kabat & Mayer (1948). With this technique less than 100 μg. N can be determined with an accuracy of ± 1–2 μg.

When precipitates containing a radioactive antigen and unlabelled antibodies are analysed, the radioactivity determination enables the amount of antigen to be calculated, and the total N less the antigen N gives the amount of antibody N. No allowance has been made in these experiments for any complement N carried down by the specific precipitate, for this amount is very small; furthermore, all the antisera used were filtered through a Berkefeld filter, which removes some, at least, of the components of complement.

Phosphorus determinations. Total P was determined by King's (1932) modification of the method of Fiske & Subbarow (1925). 60–70% of the P of most lipovitellin preparations can be extracted by treatment with an ethanol-ether mixture (7:3 by vol.) (Francis & Wormall, 1950), and for the purposes of this paper this fraction is called lipid P, and that not extracted, the vitellin P.

Total iodine determinations. These were made by the method of Shahrokh (1943). Under the conditions of our experiments we have found this method satisfactory for the determination of 10–100 μg. of I_2, with an accuracy of ± 1 μg.

Calculation of molecular ratios. It was not practicable to determine the molecular weights of the iodinated proteins and other antigens and antibodies used in the tests. In calculating the molecular ratios recorded in this paper, the following molecular weights have been used: ovalbumin, 40,500 (Svedberg & Eriksson-Quensel, 1935–6); antibodies in rabbit sera, 165,000 (for the references, see Kabat & Mayer, 1948, p. 168); human serum globulins, 165,000. Similar values, increased to allow for the amount of I_2 introduced, have been used for the iodinated proteins. The N content of ovalbumin has been taken as 15·76% (Chibnall, Rees & Williams, 1943) and that of antibodies and all other proteins studied as 16%.

Radioactivity determinations. The Geiger-Müller counter system described by Banks (1946) was used for these determinations. Side window or bell-type end window counters have been used for most of the ^{131}I measurements and generally for determinations on solid preparations. The 'liquid counter' described by Veall (1948) was used on solutions and values obtained with ethanol-ether solutions were corrected for specific gravity differences.

Determination of ^{131}I and ^{32}P in the same precipitate. In some of our experiments we have used ^{32}P to label the antigen (lipovitellin) and ^{131}I to label the antibodies, and it was necessary to devise some method of determining each radioisotope in a mixture containing both. Since differential counting methods, using β- and γ-ray counters, proved unsatisfactory, the determinations were effected by measuring the total radioactivity of samples before and after removing the ^{131}I.

A sample (1 ml.) of the solution of the specific precipitate was heated with $KClO_3$ (about 0·5 g.) and approx. 20 N-H_2SO_4 (2 ml.), to convert all the I_2 to iodate. After addition of 0·1 N-KIO_3 (1 ml.) as carrier, the I_2 was liberated by adding excess of 1% KI (2·0 ml.) and removed by two extractions with $CHCl_3$. Since the remaining KI was radioactive, owing to exchange between iodide ion and I_2, it was then removed by the addition of a slight excess of 0·1 N-KIO_3 and two further extractions with $CHCl_3$. No significant further exchange of ^{131}I apparently takes place at this stage, for there is no interchange between radioactive I_2 and non-radioactive iodate under these conditions (cf. also Perlman, Chaikoff & Morton, 1941). The radioactivity of the deiodinated material is a measure of the ^{32}P, and the difference between this activity and the activity of the original material is a measure of the ^{131}I. A small correction (7–10 counts/min. in the liquid counter) had to be made in the values for the deiodinated material, to compensate for the natural radioactivity of the ^{40}K of the $KClO_3$. When the $KClO_3$ was replaced by $NaClO_3$ there was considerable spurting during the incineration, and it was decided, therefore, to continue to use $KClO_3$.

Determinations made with mixtures of proteins, some labelled with ^{32}P and some with ^{131}I, gave satisfactory results. The agreement between the observed and theoretical values was very good for

Table 1. *Determination of ^{32}P and ^{131}I in mixtures of proteins labelled with the two isotopes*

	^{32}P		^{131}I	
Mixture	Added (counts/min.)	Found (counts/min.)	Added (counts/min.)	Found (counts/min.)
A	414	427	101	97
B	372	369	88	92
C	597	599	279	301

^{32}P, but not quite so good for ^{131}I, possibly because the latter determination is made by difference. The results of a few recovery experiments of this type, with mixtures of ^{32}P-containing lipovitellin and ^{131}I-containing rabbit serum proteins, are given in Table 1.

MATERIALS

Antigens

All the antigens were used immediately after preparation or were treated with 0·01% merthiolate and stored in a refrigerator.

Ovalbumin. Solutions of thrice crystallized hen ovalbumin in 0·9% (w/v) NaCl were used.

Serum globulins. The mixed serum globulins were precipitated by addition of 1 vol. of saturated $(NH_4)_2SO_4$ solution. The precipitate was dissolved in 0·9% NaCl (1 vol.) and reprecipitated with $(NH_4)_2SO_4$; the precipitate was then dialysed against 0·9% NaCl at 0–5° until the external solution gave no reaction with Nessler's reagent. The horse serum used for a few of the early experiments was 'Normal Horse serum no. 2' (Burroughs Wellcome & Co.). We subsequently learned that this preparation is heated at 58° for about 2 hr. before issue, and in all our later experiments we used fresh unheated normal horse serum.

Lipovitellin. ^{32}P-containing lipovitellin was prepared, as described by Francis & Wormall (1948), from the eggs laid by hens injected with ^{32}P-containing inorganic phosphate; the final solution contained 125·5 mg. total P and 48 mg. vitellin P/100 ml. A similar non-radioactive preparation was used for immunization.

Iodinated proteins (general). Various iodination methods were used, but nearly all involved iodination in ammoniacal solution by a modification (Wormall, 1930) of the method of Blum & Strauss (1923). This method, which is particularly suitable for immunological investigations, is not completely satisfactory for use with radioactive iodine, since it involves loss of ^{131}I as iodide. In earlier experiments, not reported here, where it was necessary to obtain the maximum utilization of ^{131}I, this loss was avoided by using a solution of I_2 in ether.

Iodinated ovalbumin (containing ^{131}I). KI (0·4 ml. of 10% solution) was added to 150 μc. of ^{131}I as NaI solution (0·1 ml.) followed by 0·1 N-KIO_3 (1·43 ml.), and the I_2 was liberated by adding 0·1 N-HCl (1·43 ml.); the KI present was sufficient to keep the liberated I_2 in solution. This solution was added, dropwise with shaking after each addition, to a mixture of a 2·5% (w/v) solution of ovalbumin (2 ml.) and 5 N-NH_3 (1 ml.) at room temperature. The iodinated ovalbumin was precipitated by adding acetic acid (5 N followed by N) to give maximum precipitation. The precipitate was dissolved in 0·9% NaCl at pH 7·5, and three further precipitations with acetic acid were carried out, the final supernatant solution being then free from detectable I_2 or iodide. This iodinated ovalbumin solution (5 ml.) in 0·9% NaCl, with 2 N-Na_2CO_3 added to bring the pH to 7·5, contained 135 mg. N and 74·6 mg. I/100 ml.; these values correspond to an iodine content of 8·1% for the iodinated ovalbumin. About one-ninth of the ^{131}I used for the iodination was present in the iodinated ovalbumin.

Iodinated human serum globulins (containing ^{131}I). The method used was as described for the preparation of ^{131}I-containing iodinated ovalbumin. The final solution, in 0·9% NaCl at pH 7·5, contained 110 mg. N and 69·4 mg. I/100 ml.; this corresponds to an average I content of 9·2% for the iodinated globulins.

Iodinated ovalbumin (non-radioactive). A solution of 0·1 N-I_2 in KI (7 ml.) was gradually added to a 2·5% solution of ovalbumin (10 ml.) plus 5 ml. 5 N-NH_3, free I_2 being detectable 5 min. after the last addition of the I_2 solution. The iodinated ovalbumin was separated and purified by precipitation with dilute acetic acid and resolution in 0·9% NaCl at pH 7·5 (four precipitations in all) as described above. The final product contained 7·3% I (based on I and N determinations).

Iodinated horse serum globulins (non-radioactive). A solution of the mixed globulins was iodinated by the method described for non-radioactive iodinated ovalbumin. Based on N and I determinations the iodinated globulins contained about 10% of I.

Iodinated rabbit serum proteins (non-radioactive). For a few precipitin tests we used, as test antigen, the iodinated mixed albumins and globulins of rabbit serum. Serum (7·5 ml.) was mixed with water (7·5 ml.) and 5 N-NH_3 (7·5 ml.) and the proteins were iodinated, and the products precipitated, as described for non-radioactive iodinated ovalbumin. The final solution, in 0·9% NaCl and at pH 7·5, contained 299 mg. N and 269 mg. I/100 ml., corresponding to an average I content of 12·6% for the mixed iodinated serum proteins.

Antibodies

All the antisera used in these experiments were obtained by immunizing four or five rabbits with each antigen (lipovitellin or iodinated horse serum globulin). A few antisera were prepared by injecting the antigen intraperitoneally at intervals of 8–10 days, but most of the best sera were obtained in response to intramuscular injections of the alum-precipitated proteins (Proom, 1943). Potent antisera were frequently obtained 9–10 days after the first injection of the alum-precipitated antigen, and most of the remaining rabbits of these groups gave good antibody response after the second or third injection. For some experiments the serum of one rabbit was used, but the antisera from each group of rabbits were usually pooled.

The antisera were filtered through Berkefeld filters, and kept either in sterile tubes at 0–4° or in sealed tubes after freeze-drying in the apparatus described by Greaves (1946). No significant difference was observed between the serological properties of the filtered and those of the freeze-dried antisera to iodinated proteins. But since there are differences in the case of preserved antisera to lipovitellin (Francis & Wormall, 1950), it is considered advisable to note here the mode of preservation of each of the antisera used in the experiments referred to in the tables and figures of this paper: namely, filtered and stored at 0–4° (Tables 2 and 3, Figs. 1 and 2), filtered and freeze-dried (Table 4).

Globulin fraction of antiserum. The total globulins of some antisera were separated by adding 1 vol. of saturated $(NH_4)_2SO_4$ solution. The precipitate was dissolved in 0·9% NaCl, reprecipitated by $(NH_4)_2SO_4$, and dialysed against 0·9% NaCl at 0–4°. Some of these globulin fractions were iodinated as described below.

Iodinated antibodies. Preliminary tests with ordinary I_2 indicated that introduction of 1% I_2 into various precipitating antibodies caused no serious reduction in their capacity to precipitate the corresponding antigen; indeed this precipitating power is not completely abolished when

3% or sometimes appreciably more I_2 is introduced. Quantitative experiments with ^{131}I, to be reported later, have fully confirmed this observation. For tracer experiments, when radio-iodine of high specific activity is available, very much smaller amounts of I_2 are adequate, but for reasons given later we have usually used sufficient I_2 to introduce 0·7–1·0% into the antibody proteins. The iodination was effected in the presence of NH_3, but in order to avoid destruction of antibody by this base, the amount of NH_3 used was considerably less than that used in the iodination of antigens.

A solution of I_2 in KI (19 mg. free I_2) containing 121 μc. ^{131}I was prepared as described above. This solution was then added, dropwise and with gentle shaking, to a mixture of the total globulins (22·5 ml. of solution containing 0·83 g. of protein) of an antilipovitellin serum and 5 N-NH_3 (4·0 ml.). The mixture was kept at room temperature for 15 min. after the addition of all the I_2 solution, and 5 N-acetic acid was then added to bring the pH to 7·5. The solution was dialysed against 0·9% NaCl at 0–4°, with a glass marble in the dialysing sac to permit mixing of the contents at intervals. The dialysis was continued until all diffusible radioactive material had been removed, and the solution of iodinated antibody globulin, diluted to 45 ml. with 0·9% NaCl, was used for the experiments described in Table 4. These labelled globulins contained 0·88% I, and gave a count of about 800/min./mg. of protein N in a liquid counter (or about ten times as much with a bell-type counter) when the analyses of the precipitates were carried out.

In later experiments, using the iodinated γ-globulin fraction of an antiserum, it has been found that complete removal of loosely attached ^{131}I can be more satisfactorily achieved by dialysing overnight and then precipitating the proteins several times with 12% (w/v) Na_2SO_4.

RESULTS

Iodinated protein antigen-antibody precipitates

The reaction between several iodinated protein antigens and antibodies to iodinated horse serum globulins was studied over a wide range of antigen concentration. In the experiments recorded in Figs. 1–3 the antigens were labelled with ^{131}I and the amount of antibody in the precipitate was calculated from the total N content, allowing for the amount of antigen N present. The iodinated protein antigens used in the precipitin tests were of different origin from those used for immunization, and this use of heterologous antigens makes it possible for the precipitin reaction to be confined largely to a reaction between the antibody and those parts of the antigen molecule containing diiodotyrosine.

Mixtures of antigen (iodinated ovalbumin or iodinated human serum globulin, both containing ^{131}I) and an antiserum to iodinated horse serum globulin (1–2 ml.) were made up with 0·9% NaCl to 5 or 6 ml. (globulin and albumin series respectively), and were kept at 37° for 3 hr. and subsequently at 0–5° for 90 hr. The centrifuged precipitates were washed three times with 2 ml. ice-cold 0·9% NaCl, dissolved in water plus a few drops 0·5 N-NaOH, and made up to 3 ml. Duplicate determinations of total N and radio-iodine were made on 1 and 0·25 ml. samples respectively.

The results of typical experiments (Figs. 1 and 2) were generally similar to those recorded for homologous antigen-antibody systems such as that between ovalbumin and anti-ovalbumin (cf. reviews by Boyd, 1947, and Kabat & Mayer, 1948). The maximum amount of antibody precipitated by the iodinated human serum globulin was, as was anticipated, considerably greater than that occurring with

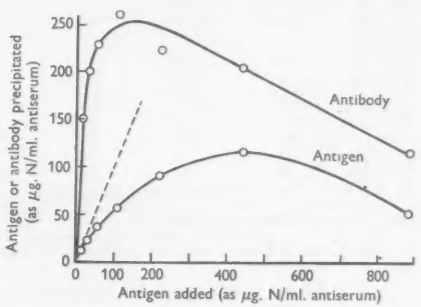

Fig. 1. Composition of specific precipitates obtained with iodinated human serum globulin and a rabbit antiserum to iodinated horse serum globulin. The broken lines in this figure and in Fig. 2 correspond to complete precipitation of the antigen.

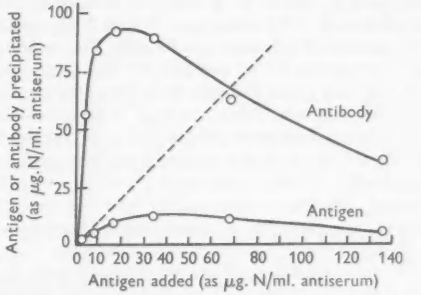

Fig. 2. Composition of specific precipitates obtained with iodinated ovalbumin and a rabbit antiserum to iodinated horse serum globulin.

the less closely related iodinated ovalbumin. Moreover, except possibly in regions of large antibody excess, the whole of the added heterologous antigen was not precipitated by the antibodies. The molecular ratio of antibody to antigen in the precipitates obtained with iodinated human serum globulin ranged from 15 in the region of antibody excess to 2·5 in the zone of antigen excess. The corresponding range for precipitates obtained with iodinated ovalbumin as antigen was 4·5–1·5 (Fig. 3).

For comparison typical results of some closely related experiments with a non-radioactive iodinated ovalbumin are given in Table 2. To allow a complete

duplicate analysis of the precipitates, it was necessary to use more antiserum and antigen in these experiments than was needed in the experiments with the radioactive antigen; e.g. in the experiment

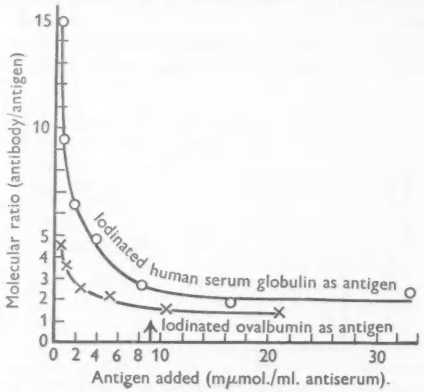

Fig. 3. Antibody-antigen molecular ratios in specific precipitates obtained with a rabbit antiserum to iodinated horse serum globulin. With all mixtures corresponding to points to the left of the arrow, excess antibody was detectable in the supernatant solution.

described in Table 2, 4–8 ml. of antiserum were added to each tube, as compared with 2 ml. with the ^{131}I-containing iodinated ovalbumin. The molecular ratio of antibody to antigen in the precipitates referred to in Table 2 ranged from 5·0 in the region of antibody excess to 1·9 in the zone of antigen excess.

In order to eliminate as completely as possible the serological effect of the original protein used for the preparation of the immunizing iodinated protein antigen, precipitin tests similar to those described above were made with iodinated mixed rabbit serum proteins as the test antigen. The specific reaction in this case was probably confined entirely to one between the antibodies and the 3:5-diiodotyrosine groups of the antigen. Although this experiment was not made with radio-isotope-labelled reagents the results are included here (Table 3), since there are little quantitative data available in relation to serological precipitates with labelled rabbit serum proteins used as the test antigen in precipitin reactions with rabbit antisera. In view of the fact that the test antigen was a mixture of iodinated globulins and albumins, it would be unwise to attach great significance to antibody/antigen molecular ratios for the precipitates obtained, but it is interesting to note that if an arbitrary average molecular weight of 100,000 is assumed for the proteins of the test antigen, the molecular ratio in the precipitates ranged from 4·4 (antigen excess zone) to 12·6 (antibody excess).

Composition of lipovitellin-antilipovitellin precipitates

In several experiments we separately labelled the antigen and antibody with different radioisotopes. In one series of experiments we used ^{32}P-containing lipovitellin as the antigen, and the corresponding antibodies were labelled with ^{131}I. By using radioiodine of fairly high specific activity we were able to avoid significant loss of serological activity of the antibodies. Since the antigen contained lipid P as well as protein (vitellin) P, the serological precipitates were analysed for these two P fractions, as well as for antibody. Preliminary tests showed that ^{131}I could be quantitatively removed from the antigen-antibody complexes without loss of ^{32}P, as is described earlier in this paper. Briefly, the radioactivity measurements on each precipitate involved determinations on (*a*) the material extracted by ethanol-ether (lipid P), (*b*) the extracted precipitate (vitellin

Table 2. *Composition of precipitates obtained with iodinated ovalbumin and antisera to iodinated horse serum globulin*

(Mixtures of antigen (iodinated ovalbumin) solution and antiserum (4 ml. in tubes 1 and 2; 8 ml. in tubes 3, 4 and 5) were made up with 0·9% NaCl to 9 ml. total volume in each case, incubated at 37° for 3 hr. and kept at 0–5° for a further 90 hr. The centrifuged precipitates were washed twice with 0·9% NaCl (6 drops each time), dissolved in water plus a few drops 0·5 N-NaOH, and made up to 15 ml. Duplicate total N and I determinations were made on 1 and 6 ml. samples respectively, of these solutions.)

	Antigen added		Tests† on supernatant solution		Composition of precipitate				
Tube no.	N* (μg.)	I* (μg.)	Antigen present	Antibody present	Total N (μg.)	Total I (μg.)	Antigen N (μg.)	Antibody N (μg.)	Mol. ratio antibody/ antigen
1	47·8	23·6	tr.	0	272	15·2	30·7	241	1·9
2	23·9	11·8	tr.	f.tr.	242	8·4	17·0	225	3·2
3	11·9	5·9	0	±	151	5·1	10·3	141	3·4
4	9·55	4·72	0	+	149	3·9	7·9	141	4·4
5	7·96	3·94	0	+	122	2·8	5·7	116	5·0

* All the N and I values in this table are expressed as μg./ml. of antiserum used.

† Tested by the addition of antiserum and antigen to separate samples; 0, none detectable; f.tr., faint trace; tr., trace; ±, moderate and +, strong, indicate the extent of precipitation.

Table 3. *Composition of precipitates obtained with iodinated rabbit serum proteins and rabbit antisera to iodinated horse serum globulins*

(The test antigen (5 ml. of a 0·0043–0·036% solution of iodinated rabbit serum proteins) was mixed with antiserum (4 ml.) and the mixture kept for 3·5 hr. at 37° and then overnight in the refrigerator. The centrifuged precipitates were washed twice with ice-cold 0·9% NaCl solution (0·5 ml. each time) and dissolved in water with the aid of a few drops of 0·5 N-NaOH. The solutions were made up to 15 ml. and duplicate N and I determinations were made on 1 and 6 ml. samples respectively.)

	Antigen added		Tests† on supernatant solution		Composition of precipitate				
Tube no.	N* (μg.)	I* (μg.)	Antigen present	Antibody present	Total N (μg.)	Total I (μg.)	Antigen N (μg.)	Antibody N (μg.)	Antibody N/antigen N
1	74·8	67·3	+	0	178	19·2	21·4	157	7·3
2	37·4	33·6	+	0	213	19·6	21·8	191	8·8
3	29·9	26·9	±	0	184	16·1	17·9	166	9·3
4	18·7	16·8	tr.	f.tr.	152	10·6	11·8	140	11·9
5	14·9	13·4	f.tr.	f.tr.	105	5·5	6·1	99	16·2
6	9·0	8·1	f.tr.	±	32·6	1·4	1·5	31	20·7

* All the N and I values in this table are expressed as μg./ml. of antiserum used.

† Tested by the addition of antiserum and antigen to separate samples; 0, none detectable; f.tr., faint trace; tr., trace; ±, moderate and +, strong, indicate the extent of precipitation.

Table 4. *Composition of lipovitellin-antilipovitellin precipitates*

(Antigen: lipovitellin (containing ^{32}P); antibodies: the globulin fraction (labelled with ^{131}I) of an antilipovitellin serum. All the weights given in this table are in terms of μg./ml. of antiserum used. Experimental details are given in the text. L = lipid (phospholipin); V = vitellin; AB = antibody.)

Antigen		Composition of the precipitate					
					Mol. ratios*		
Lipid P (μg.)	Vitellin P (μg.)	Lipid P (μg.)	Vitellin P (μg.)	Antibody N (μg.)	(L/V)†	(AB/V)	(L/AB)
77·5	48	26·7	5·8	217	136	1·30	104
103	64	30·0	6·9	253	129	1·27	102
129	80	37·2	7·6	270	147	1·24	119
155	96	40·0	7·6	257	155	1·16	134

* Assuming a molecular weight of 778 for phospholipin (palmito-oleo-lecithin; 3·99% P), and an arbitrary value of 100,000 for vitellin (0·92% P).

† The corresponding L/V ratio for the lipovitellin used in this experiment was 48.

P plus antibody I), and (*c*) the extracted precipitate after it had been de-iodinated (vitellin P).

Samples (6 ml.) of a solution of the iodinated globulins of an antiserum to lipovitellin were each treated with NaCl (0·6 g.) and a solution of lipovitellin (5 ml. of the stock solution suitably diluted with 10% NaCl). After being kept for 3 hr. at 37° and then overnight at about 4°, the mixtures were centrifuged and the supernatant solutions removed. The inside walls of the tubes were washed with 10% NaCl (5 drops) and the tubes were again centrifuged. Each precipitate was then washed three times with ethanol-ether (7:3 by vol.; 4 ml. each time). The extract was diluted to 15 ml. and the radioactivity of the solution was determined (using a liquid counter) and compared with that of the phospholipins of the original antigen. The lipid-free precipitates were treated with water (2 ml.) and 0·5 N-NaOH (12 drops); they were left overnight at 37° and subsequently heated for 2–3 hr. in a boiling-water bath to complete the solution of the precipitates. Each solution was then diluted to 5 ml. and samples used for radioactivity determinations before and after de-iodination. Control experiments were made to determine how much lipovitellin was carried down by an unrelated antigen-antibody precipitate (mustard gas sulphone-treated ovalbumin and its antiserum) and similar tests were made with the labelled antilipovitellin. The appropriate small allowances were made for this non-specific adsorption.

The results of this experiment are given in Table 4, which also includes some provisional calculated values for the relative numbers of molecules of lipid, vitellin and antibody present in each of the precipitates analysed. These molecular ratios should be regarded as having only relative significance where they involve vitellin molecules, since no information is available about the molecular weight of this insoluble protein.

DISCUSSION

Antigen-antibody precipitates can be analysed in various ways, depending largely on the nature of the antigen. Where the antigen contains no readily determinable 'unusual' element or group (e.g. iron

or dyestuff), the analysis largely depends on total N determinations. The method developed by Heidelberger and his colleagues (see Heidelberger & Kendall 1935; Heidelberger, 1939, 1946) and successfully used in the study of many different serological precipitates, requires, for systems containing an ordinary protein antigen, the following: (*a*) a determination of the total N content of the precipitate, and (*b*) tests to show that all the antigen has been precipitated (equivalence and antibody excess zones), or, for the antigen excess zone, a complicated analysis of the supernatant solution to determine how much antigen it contains.

With antigens and antibodies labelled with radio-isotopes, the analysis of specific precipitates is comparatively simple; moreover, the method is equally applicable in the antigen excess zone. When a protein antigen is used and either the antigen or the antibody is labelled with a radio-isotope, the composition of the precipitate can be calculated from its radioactivity and total N content; if the isotope preparation is of high specific activity, the amount of precipitate required for the complete analysis is only very slightly greater than that needed for the total N determination. When both antigen and antibody are labelled, with different radio-isotopes which can be separately determined in mixtures containing both, the complete analysis can often be made on extremely small amounts of precipitate. Usually, however, there is no need for this strict economy of antigen or antibody, and in none of the experiments described here was an attempt made to work with minute amounts of material.

As antigens we have used ovalbumin, serum globulins and the mixed serum proteins containing ^{131}I, and biologically prepared ^{32}P-containing lipovitellin; in other experiments, not described here, protein antigens have been labelled by treatment with mustard gas sulphone (di-2-chloroethyl sulphone) containing ^{35}S. Both ^{131}I and ^{35}S are suitable labels for immunological studies, and, with radioactive material of high specific activity, iodine atoms or 'sulphone groups' can be introduced in such small amounts that there is no significant change in the immunological specificity of the antigen; alternatively, many of these groups may be introduced into the protein molecule and a labelled protein of new specificity obtained.

Our experiments with biologically prepared isotopically labelled protein antigens have been confined so far to those with lipovitellin and vitellin. The presence of the label in both the protein and phospholipin of ^{32}P-containing lipovitellin sometimes leads to complications, and moreover lipovitellin is a complex which may vary significantly from one preparation to another. Precise chemical information about this complex is rather scanty, but recently Mecham & Olcott (1949) have described the isolation of a new water-soluble egg yolk phosphoprotein, which they named 'phosvitin' and which may account for 60–79% of the phosphoprotein P of egg yolk; a typical 'phosvitin' preparation had a P/N ratio of 0·815, compared with 0·14–0·17 for our lipovitellin preparations. Further investigations on the immunological properties of vitellin and lipovitellin appear desirable in the light of these observations.

Lipovitellin, although useful for the study of antibodies against lipids (Francis & Wormall, 1950), is not an ideal antigen for quantitative studies on specific precipitates. For experiments of this type, particularly for the study of serological reactions on a molecular basis, homogeneous and, if possible, crystalline protein antigens should be used. We have, therefore, used crystalline ovalbumin for many of our quantitative investigations. For other experiments, however, we have used the serum globulins and very occasionally mixed serum proteins, since these are sometimes more suitable than are proteins which are more 'foreign' to the animal body. For example, when chemically altered protein antigens are prepared from serum globulins, particularly those from the same species of animal used for the immunization, the parts of the antigen molecule involved in the precipitin reaction are largely, if not entirely, those containing the introduced group. Also, the zone of maximum precipitation is usually broader when unaltered or chemically altered mixed globulins, rather than crystalline proteins, are used as test antigens.

For the labelling of antibodies, we have found ^{131}I very satisfactory, and up to 1% of iodine can be introduced into the antibodies studied without serious loss of precipitating power. Much less is sufficient for tracer purposes, but we usually introduced 0·7–1·0% into the antibody molecule, equivalent to an average of 4·5–6·5 mol. of iodine (mainly or entirely in 3:5-diiodotyrosine groups) per mol. of antibody. With this degree of iodination it is highly probable that practically all the antibody molecules contain at least one tracer group (cf. Butement, 1948). If much less iodine is introduced the possibility of preferential precipitation of unlabelled, relative to labelled, antibody molecules cannot be excluded. No proof is yet available that the percentage of iodine in the antibody molecules in the precipitate is the same as that in the original iodinated antibodies, but this is probably true when most of the antibody is precipitated; whether it is true in the antibody excess zone we intend to study later. Pressman & Keighley (1948), Pressman (1949) and Butement (1948) have also used ^{131}I for labelling antibodies, and they have introduced amounts of iodine similar to those used here.

The molecular composition of specific precipitates has usually been studied with homologous systems,

e.g. ovalbumin-antiovalbumin, and there are relatively few quantitative observations on heterologous systems, particularly with chemically altered protein antigens. Our results are generally similar to those recorded for the ovalbumin and other homologous systems, but the zone of maximum precipitation for the heterologous system appears to be somewhat broader. Moreover, since the heterologous antigen is not perfectly 'adapted' to the antibodies, it is not completely precipitated except possibly in the region of extreme antibody excess (Figs. 1 and 2). The ratios of antibody to antigen in the precipitates are of the same order with heterologous and homologous systems (Fig. 3, and cf. Boyd, 1947, p. 243, and Landsteiner, 1945). None of the serological precipitates we have analysed contained fewer antibody than antigen molecules.

The lipovitellin-antilipovitellin precipitates contained considerably more phospholipin in relation to vitellin than did the lipovitellin used as antigen. Whatever arbitrary value is used for the molecular weight of the lipid-free protein vitellin, the molecular ratio of phospholipin to vitellin in the precipitates was about three times that for the lipovitellin (Table 4). These results provide further evidence of the capacity of rabbit antilipovitellin sera to precipitate phospholipins (Francis & Wormall, 1950).

SUMMARY

1. Quantitative studies on the precipitin reaction have been made with antigens and antibodies labelled with radioactive isotopes. This technique greatly simplifies the study of the composition of serological precipitates particularly in the antigen excess zone.

2. The results are given for analyses of serological precipitates containing antigens labelled with ^{32}P (lipovitellin) or ^{131}I (iodinated globulins and ovalbumin), and antibodies labelled with ^{131}I.

3. Up to 1 % of iodine can be introduced into the proteins of many precipitating antisera without serious loss of their precipitating power.

4. Lipovitellin-antilipovitellin precipitates contained 100 or more molecules of phospholipin per molecule of antibody. The ratio lipid P:vitellin P in these precipitates was 4·3–5·3:1, compared with 1·6:1 for the sample of lipovitellin used. These results support the view previously put forward (Francis & Wormall, 1950) that antilipovitellin sera contain antibodies capable of precipitating phospholipins.

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The Biological Action of Substances Related to Thyroxine

1. THE EFFECT OF *n*-ALKYL 3:5-DIIODO-4-HYDROXYBENZOATES ON OXYGEN CONSUMPTION IN MICE

By MARY M. SHEAHAN, J. H. WILKINSON and N. F. MACLAGAN
Department of Chemical Pathology, Westminster Medical School (University of London), London, S.W. 1

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Since the principle of competitive inhibition in chemotherapy was introduced by Woods (1940), several attempts have been made to prepare compounds with an inhibitory action against thyroxine. The first report of direct antagonism was that of Woolley (1946) who observed that a number of ethers of *N*-acetyldiiodotyrosine exhibited antithyroxine activity in amphibian metamorphosis. Harington (1948) suggested that it might be possible to distinguish between the effects of thyroxine on the pituitary and on the peripheral tissues. In an attempt to inhibit one or other of these effects he prepared the thio-ether analogue of thyroxine, but this proved to have thyroxine-like activity when tested in tadpoles. Also using tadpoles as test animals, Frieden & Winzler (1949) described the inhibitory action of a number of compounds, including the benzyl ethers of diiodotyrosine and 3:5-diiodo-4-hydroxybenzoic acid, and produced evidence to support their view that this effect was due to true competitive inhibition. Cortell (1949) has demonstrated the antithyroxine properties of 2′:6′-diiodothyronine in rats by the goitre prevention method.

The desirability of extending the methods of studying thyroxine inhibition in mammalia led us to develop a technique for determining the effects of such compounds on the rise of oxygen consumption produced by thyroxine in mice (Maclagan & Sheahan 1950). By this method one of the compounds described by Frieden & Winzler (1949), 4-benzyloxy-3:5-diiodobenzoic acid, was found to be active, and subsequently, the dimethylacetal of 3:5-diiodoanisaldehyde (Wilkinson, 1949) also gave positive results (Maclagan, Sheahan & Wilkinson, 1949). A brief account of the effects of a series of *n*-alkyl 3:5-diiodo-4-hydroxybenzoates has already appeared (Wilkinson, Sheahan & Maclagan, 1950), and it is the purpose of the present paper to discuss more fully the results obtained and also to extend the study to a number of related substances.

Of the two methods available for the preparation of the *n*-alkyl 3:5-diiodo-4-hydroxybenzoates, that involving iodination of the *n*-alkyl *p*-hydroxybenzoates was preferred, since the alternative procedure of esterifying the diiodo- acid invariably resulted in some decomposition, and in consequence the products proved difficult to purify. Most of the required alkyl hydroxybenzoates have been described by Cavill & Vincent (1947). Several diiodophenols were prepared by Paal (1895) by treatment of the phenols with two equivalents of iodine in aqueous ethanolic solution in the presence of the calculated quantity of iodic acid. As the acid conditions of this method were liable to cause hydrolysis of the esters, we replaced the iodic acid by an equivalent quantity of potassium iodate, and, by this means, the eight *n*-alkyl esters from methyl to *n*-octyl were obtained in good yields.

EXPERIMENTAL

Chemical methods

All melting points are uncorrected. Micro-analyses were by Drs Weiler and Strauss, Oxford.

Preparation of n-*alkyl* p-*hydroxybenzoates.* The alkyl *p*-hydroxybenzoates were prepared by the method of Cavill & Vincent (1947). The *n*-octyl ester, not described by these authors, was obtained by heating a mixture of *p*-hydroxybenzoic acid (13·8 g.), *n*-octanol (45 ml.), toluene (50 ml.) and H_2SO_4 (1 g.) under reflux for 3 hr., a McIntyre still head being employed for water separation. The cooled mixture was neutralized with NaOH, acidified with acetic acid, and steam distilled. The residue was extracted with ether, dried (Na_2SO_4) and distilled. n-*Octyl* p-*hydroxybenzoate* was obtained as a pale-yellow oil, b.p. 170–172°/0·1 mm., which solidified to a colourless waxy solid, m.p. 47–48°. Yield: 15·7 g. (63 %). (Found: C, 72·6; H, 8·8. $C_{15}H_{22}O_3$ requires C, 72·6; H, 8·8 %.)

Iodination of n-*alkyl* p-*hydroxybenzoates.* The alkyl hydroxybenzoate (0·1 g.mol.) was dissolved in the corresponding alcohol (100 ml.) and treated with I_2 (25·4 g.). A solution of KIO_3 (7·1 g.) in water (80 ml.) was gradually added to the boiling mixture which was then heated on a steam bath for 2 hr. The alcohol was removed by steam distillation, traces of unreacted I_2 were destroyed with $Na_2S_2O_3$, and the product collected by filtration. By this means the following compounds were prepared in yields from 80 to 95 %.

Methyl 3:5-diiodo-4-hydroxybenzoate crystallized from the reaction mixture, and, in this case, steam distillation proved unnecessary. Recrystallization from methanol gave colourless needles, m.p. 165°, identical with a sample prepared by esterification of 3:5-diiodo-4-hydroxybenzoic acid. Wheeler & Liddle (1910) report m.p. 165°.

Ethyl 3:5-*diiodo-4-hydroxybenzoate*. Colourless needles from ethanol, m.p. 124°. (Found: C, 26·2; H, 2·2; I, 61·4. $C_9H_8O_3I_2$ requires C, 25·8; H, 1·9; I, 60·8%.)

n-*Propyl ester*. Colourless needles from benzene, m.p. 123°. (Found: C, 27·2; H, 2·6; I, 59·2. $C_{10}H_{10}O_3I_2$ requires C, 27·8; H, 2·3; I, 58·8%.)

n-*Butyl ester*. Colourless prisms, from 90% methanol, m.p. 90°. (Found: C, 29·6; H, 2·5; I, 56·7. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·0%.)

Ethanol was employed as solvent for the iodination of the following esters:

n-*Amyl ester*. Colourless needles from benzene-light petroleum (b.p. 60–80°), m.p. 78°. (Found: C, 31·0; H, 2·8; I, 57·5. $C_{12}H_{14}O_3I_2$ requires C, 31·3; H, 3·05; I, 55·2%.)

n-*Hexyl ester*. Colourless waxy needles from light petroleum (b.p. 80–100°), m.p. 70–71°. (Found: C, 33·2; H, 3·4; I, 53·7. $C_{13}H_{16}O_3I_2$ requires C, 32·9; H, 3·4; I, 53·6%.)

n-*Heptyl ester*. Colourless glistening waxy plates from 90% methanol, m.p. 68°. (Found: C, 34·8; H, 3·75; I, 52·4. $C_{14}H_{18}O_3I_2$ requires C, 34·5; H, 3·7; I, 52·1%.)

n-*Octyl ester*. Colourless waxy needles from 90% methanol, m.p. 65°. (Found: C, 36·2; H, 4·3; I, 50·2. $C_{15}H_{20}O_3I_2$ requires C, 35·9; H, 4·0; I, 50·6%.)

n-*Butyl* 3:5-*diiodosalicylate* was prepared similarly from *n*-butyl salicylate. After removal of the butanol by steam distillation, the residue was extracted with ether. The extract was washed with $Na_2S_2O_3$ and water and dried (Na_2SO_4). After removal of the solvent, the residue was heated in an oil bath at 180–190°/0·1 mm. to remove a small amount of unreacted *n*-butyl salicylate. The residual oil crystallized on cooling, and recrystallization from light petroleum (b.p. 40–60°) gave the required *ester* in colourless prisms, m.p. 53–54°. (Found: C, 29·9; H, 2·7; I, 57·4. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·0%.)

n-*Butyl* 3:5-*diiodobenzoate* was obtained by heating a mixture of 3:5-diiodobenzoic acid (2·9 g.), *n*-butanol (30 ml.), toluene (30 ml.) and H_2SO_4 (1 g.) as described for *n*-octyl *p*-hydroxybenzoate. The crude product (2·99 g.) was extracted (Soxhlet) with light petroleum (b.p. 40–60°) and the extract concentrated. The brown solid which separated crystallized from light petroleum (b.p. 40–60°) in buff-coloured needles, m.p. 50°. (Found: I, 58·6. $C_{11}H_{12}O_2I_2$ requires I, 59·1%.)

n-Butyl 3:5-dibromo-4-hydroxybenzoate was prepared as described by Cavill (1945).

n-*Butyl* 3:5-*dichloro-4-hydroxybenzoate*. *n*-Butyl *p*-hydroxybenzoate (9·7 g.) was dissolved in acetic acid (50 ml.) and the solution cooled to 10°. Cl_2 was passed through the solution until it was saturated, about 2 hr. were required. The temperature was maintained at 10–15° throughout this operation. Ice water (200 ml.) was added and the precipitated solid (12·7 g., 96%) collected by filtration. The product was washed free from acid with water and dried over $CaCl_2$. The required *ester* crystallized from light petroleum (b.p. 60–80°) in colourless prims, m.p. 87–88°. (Found: C, 49·9; H, 4·6; Cl, 27·3. $C_{11}H_{12}O_2Cl_2$ requires C, 50·2; H, 4·6; Cl, 27·0%.)

The ester (0·13 g.) was dissolved in 2 N-NaOH (4 ml.) and heated under reflux for 30 min. On acidification with HCl, 3:5-dichloro-4-hydroxybenzoic acid (91 mg.) m.p. 265°, was obtained. Several values, ranging from 255° to 265°, for the melting point of this acid appear in the literature (cf. Beilstein's *Handbuch*, vol. x, p. 177).

The benzoic and *p*-hydroxybenzoic acids and their *n*-butyl esters were commercial samples purified where necessary.

Measurement of oxygen consumption

The methods used for the determination of the rate of O_2 consumption were essentially those of Maclagan & Sheahan (1950), but a number of slight modifications were made. Before treatment the mice were randomized into groups of eight. Each group remained together throughout the experiment to eliminate any restlessness that might be caused by mixing strange mice for the first time on the day upon which their O_2 consumption was to be measured. The inhibitory compounds when given alone to normal mice showed no significant alteration in metabolism and we have therefore relied on the depression of O_2 consumption in mice previously treated with thyroxine to demonstrate inhibitory effects. In each experiment two groups of untreated mice were used as controls, two were given thyroxine only and two were given thyroxine plus the compound under test.

The total dose of the compounds was administered in two parts, half being given at 11 a.m. on the first day and the remainder at 11 a.m. on the second day. A single dose of the monosodium salt of DL-thyroxine was given at 4 p.m. on the first day. O_2 consumption was measured on the third day, i.e. 48 hr. after the first injection of the compound and 43 hr. after the thyroxine injection. The compounds and the hormone were given by subcutaneous injection in suspension or solution in 0·1% (w/v) aqueous Na_2CO_3. In addition, several experiments at different dose levels were performed in which *n*-butyl 3:5-diiodo-4-hydroxybenzoate was given orally.

Each group of mice was placed in a desiccator: 0·5 hr. was allowed for the atmosphere to warm up, when the first reading was taken. Six more readings were taken at half-hourly intervals. Thus there were twelve readings for each group of treated mice, and the statistical significance of these results was analysed by student's '*t*' test.

RESULTS

A typical experiment showing the effect on oxygen consumption of thyroxine when given alone, and when given with *n*-butyl 3:5-diiodo-4-hydroxybenzoate, is illustrated in Fig. 1. It will be seen that the inhibitor produced no significant effect when given alone. Fig. 2 shows the minimum dose at which 3:5-diiodo-4-hydroxybenzoic acid and its *n*-alkyl esters were effective in reducing the oxygen consumption in thyroxine-treated mice. All the esters were more active than the acid, and the first four esters showed a high degree of potency. Of these the *n*-butyl ester was the most active, producing appreciable inhibition at 25 mg./kg. by the subcutaneous route, whilst the remaining four esters were much less active. Detailed results for the *n*-butyl ester are given in Table 1 which shows that, even at the highest dose level, inhibition was not complete, though there appears to be some relation between the degree of inhibition and the dosage. When administered orally this ester was active at a total dose of 50 mg./kg., but was devoid of activity at lower doses.

The acid produced a significant inhibition in mice at a molar ratio of 1000 : 1, in contrast to the

experience of Frieden & Winzler (1949) who, using tadpoles, found it inactive at the same molar ratio. It would seem that with this compound results obtained with one species may not be directly applicable to another.

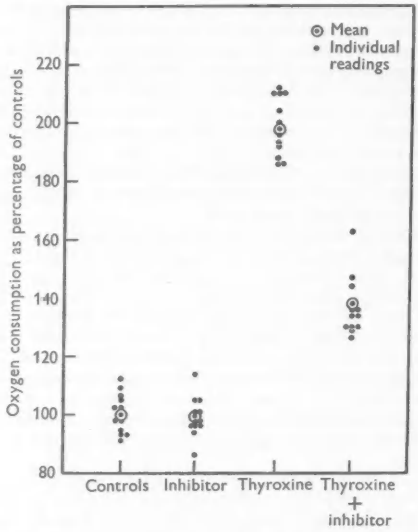


Fig. 1. Oxygen consumption of mice injected subcutaneously with thyroxine and *n*-butyl 3:5-diiodo-4-hydroxybenzoate. Measurements were taken after two doses of 25 mg./kg. of the inhibitor and 2·0 mg./kg. DL-thyroxine sodium.

Earlier work (Maclagan *et al.* 1949), in which 3:5-diiodoanisaldehyde dimethylacetal caused partial inhibition of the thyroxine effect, whilst the free aldehyde was inactive, originally led us to suppose that increase in lipophilic properties might parallel an increase in antithyroxine action. The results obtained in the course of the present work, however, indicate that the relationship between chemical constitution and biological activity is more complicated than could be accounted for by a simple partition effect between fat and water.

Replacement of the iodine atoms by other halogens or hydrogen completely abolished the activity of the *n*-butyl ester (Table 2). Replacement of the hydroxyl group by hydrogen to give *n*-butyl 3:5-diiodobenzoate also caused complete loss of activity.

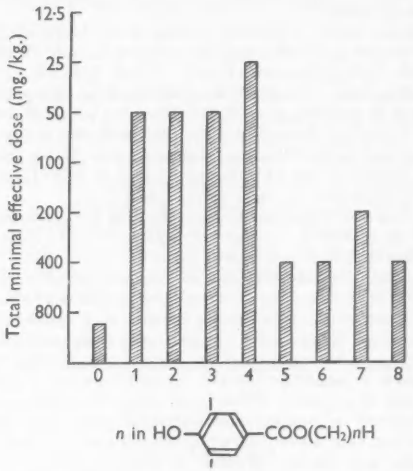


Fig. 2. The effect of variations in chain length upon antithyroxine activity of *n*-alkyl 3:5-diiodo-4-hydroxybenzoates.

A hydroxyl group in the 2-position, however, restored the antithyroxine activity to some extent, for *n*-butyl 3:5-diiodosalicylate produced appreciable inhibition. *n*-Butyl benzoate was without significant action. Neither benzoic acid nor *p*-hydroxybenzoic acid had any effect on oxygen consumption, though Keeser (1949) has reported that benzoic acid inhibited the action of thyroxine on carbohydrate metabolism in rats, and also on iso-

Table 1. *The effect of* n-*butyl* 3:5-*diiodo*-4-*hydroxybenzoate on the oxygen consumption of thyroxine-treated mice*

Dosage (mg./kg.)			Oxygen consumption as percentage of controls (Mean ± S.E.)			
Inhibitor (*I*)	DL-Thyroxine (*T*)*	Molar ratio (*I*/*T*)	+Thyroxine (*a*)	+Thyroxine +inhibitor (*b*)	Percentage inhibition $\left(\frac{a-b}{a}\times 100\right)$	Significance (Student's *t* test)
1000	1·5	1150	187 ± 5·1	128 ± 5·5	68	P 0·001
400	1·5	460	183 ± 8·5	115 ± 3·3	82	$P < 0·001$
200	1·5	230	187 ± 3·3	122 ± 4·0	75	$P < 0·001$
100	2·0	87	161 ± 3·5	117 ± 2·9	72	$P < 0·001$
50	2·0	44	199 ± 2·9	137 ± 3·0	63	$P < 0·001$
25	2·0	22	161 ± 2·8	137 ± 6·1	56	$P < 0·001$
12	1·0	20	194 ± 3·2	190 ± 3·5	4	Not significant

* The dose of thyroxine was adjusted to produce a standard response, i.e. 60–90% increase in oxygen consumption.

Table 2. *Effect of analogues of* n-*butyl* 3:5-*diiodo*-4-*hydroxybenzoate on thyroxine-treated mice*

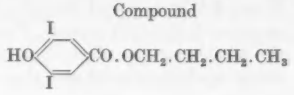
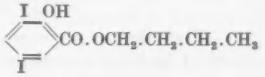
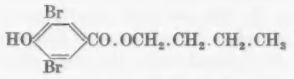
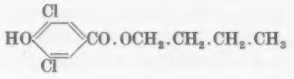
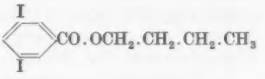
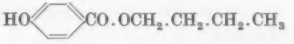
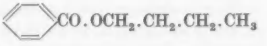
Compound	Activity
	Inhibitory at 25 mg./kg.
	Inhibitory at 1000 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 1000 mg./kg.
	Inactive at 400 mg./kg.
NaI	Inactive at 700 mg./kg.

Table 3 *Toxic and effective doses of* n-*butyl* 3:5-*diiodo*-4-*hydroxybenzoate*

	Minimum* effective dose (mg./kg.)	LD_{50} (mg./kg.)	Ratio LD_{50}/M.E.D.
Oral route	50	1500	30
Subcutaneous route	25	3500	140

* The minimum effective dose is the smallest dose which produced a statistically significant diminution of the O_2 consumption of thyroxinized mice.

lated enzyme systems. Sodium iodide in amounts equivalent to the highest tolerated doses of inhibitors was also without effect.

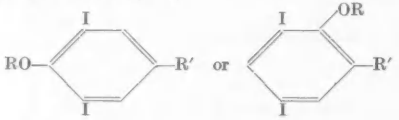
Table 3 shows the relationship between the minimal effective dose (M.E.D.) and the toxicity (LD_{50}) for *n*-butyl 3:5-diiodo-4-hydroxybenzoate by oral and subcutaneous routes. The ratio, LD_{50}/M.E.D. appears to be sufficiently high to suggest that this compound might be suitable for clinical trial. It may be noted here that the ethyl ester was substantially more toxic, producing four deaths in eighteen mice at a dosage of 1000 mg./kg. subcutaneously.

DISCUSSION

The work described above was carried out with the object of finding compounds which would antagonize the various actions of thyroxine by a process of competitive inhibition. We consider that the evidence presented, although incomplete, strongly suggests that the active compounds tested are, in fact, producing their effects in this way. The principal points in favour of this hypothesis are as follows: (1) The effect of the inhibitors is greatest at the time of maximum thyroxine effect, i.e. 2 days after the injection of thyroxine. (2) The dose of the hormone

given in our experiments is so large that a mere inhibition of endogenous thyroxine production would probably have little effect on the results. The mode of action has not, however, been completely defined by our present method of testing, which was adopted as a screening method in order to select suitable substances for further biological study. The fact that the compounds when given alone are without effect on oxygen consumption is difficult to explain, but it is well known that such a depression of metabolism is not easy to achieve in mice even with drugs of the thiouracil type.

Whatever the mechanism of the effect observed, it appears to require a fairly high degree of specificity in molecular structure, and the structures of successful compounds bear at least a superficial resemblance to that of thyroxine. Thus activity in this series of compounds is confined to those substances which contain either the group



and it is possible that the diiodophenol part of the esters discussed may compete with the same part of the thyroxine molecule for a position on some receptor surface.

We have been privileged to read the manuscript of a paper by Barker, Kiely, Dirks, Klitgaard, Wang & Wawzonek (1950) which is in general agreement with this hypothesis. These workers describe the antithyroxine effect of a series of iodophenoxyacetic acids in normal and thyroidectomized mice.

SUMMARY

1. The effect of a series of *n*-alkyl esters of 3:5-diiodo-4-hydroxybenzoic acid on the oxygen consumption of thyroxine-treated mice has been studied.

2. The methyl, ethyl, propyl and butyl esters exhibited a strong antithyroxine effect, the butyl compound being the most active. The activity was almost as great by mouth as by the subcutaneous route. The higher esters were less potent, and the acid itself showed only weak activity.

3. Variations in halogen substitution or elimination of the 4-hydroxyl group in the *n*-butyl ester completely abolished the thyroxine-inhibitory effect. *n*-Butyl 3:5-diiodosalicylate showed slight antithyroxine activity. Sodium iodide was inactive.

4. Evidence is presented in support of the view that a specific competitive inhibition of thyroxine is involved.

It is a pleasure to acknowledge the continued assistance given to this research by Glaxo Laboratories Ltd. The toxicity tests on *n*-butyl 3:5-diiodo-4-hydroxybenzoate were carried out by them.

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Metabolism of Derivatives of Toluene

6. TOLUNITRILES, BENZONITRILE AND SOME RELATED COMPOUNDS

By H. G. BRAY, Z. HYBS and W. V. THORPE

Department of Physiology, Medical School, University of Birmingham

(*Received* 8 *July* 1950)

We have suggested previously (see Thorpe, 1950) that methyl and carbamyl groups in aromatic compounds may be termed 'potential centres for conjugation', since under suitable conditions they are converted by the animal to carboxyl and the resulting acids conjugated with glycine or glucuronic acid. We are investigating the behaviour in the rabbit of other potential centres which might be converted to carboxyl, e.g. —CH_2OH, —CHO, —CN, and this present report is concerned with the nitrile group as it occurs in benzonitrile and the tolunitriles. This group might be hydrolysed to carbamyl or to carboxyl. No previous investigation of the fate of the tolunitriles appears to have been carried out.

Benzonitrile was found by Giacosa (1883) to be converted to benzoic acid to a small extent by the dog, while Baumann (1883) stated that its administration to dogs increased the excretion of ethereal sulphate. The latter also isolated small amounts of *o*- and *p*-hydroxybenzoic acids from benzonitrile urine. Adeline, Cerecedo & Sherwin (1926) were unable to detect any metabolites of benzonitrile in the rabbit. Smith & Williams (1950) found that benzonitrile was eliminated very slowly by the rabbit, about 60 % being excreted as *O*-conjugates; they claimed that small amounts were probably converted to benzoic acid and a mercapturic acid. *m*- And *p*-cyanophenols were isolated from the ethereal sulphate fraction of the urine. In the experiments now reported the effect of these four compounds on the excretion by rabbits of ether-soluble acid, ester and ether glucuronides and ethereal sulphate has been determined and several metabolites of the tolunitriles have been isolated. The fate of some of these metabolites has also been examined.

MATERIALS AND METHODS

Materials

m-Tolunitrile, *m*- and *p*-cyanophenols and *m*- and *p*-cyanobenzoic acids were prepared by the Sandmeyer reaction, starting from the corresponding amino compounds. *o*-Cyanobenzoic acid could not be obtained in this way, but was prepared from phthalimide by the method of Braun & Tcherniac (1907) in such small yield as to render it impracticable to accumulate sufficient for dosing. The acid is unstable and forms phthalamic acid on standing in water at 37° for 24 hr. *o*-Cyanophenol was prepared by the action of acetic anhydride upon salicylaldoxime (Bone, 1893). Terephthalamic acid was obtained by the action of H_2O_2 on *p*-cyanobenzoic acid (cf. the preparation of *o*-toluamide from *o*-tolunitrile, Noller, 1933). Phthalamic acid was prepared by the action of KOH on phthalimide at room temperature (Aschan, 1886). All melting points agreed with those given in the literature. Attempts to prepare *o*-cyanobenzyl alcohol by hydrolysis of *o*-cyanobenzyl chloride were unsuccessful.

p-*Cyanohippuric acid* was obtained by a Sandmeyer reaction upon *p*-aminohippuric acid. It formed colourless leaflets, m.p. 191°. (Found: C, 59·2; H, 4·0; N, 13·5; equiv. (by titration) 208. $C_{10}H_8O_3N_2$ requires C, 58·8; H, 3·9; N, 13·7 %; equiv. 204.)

Diet and dosage

The rabbits used, does weighing 2–3 kg., were maintained throughout on the standard diet of rabbit pellets previously described (Bray, Ryman & Thorpe, 1947). The nitriles were administered by stomach tube as emulsions in warm water and the acids as solutions of their Na salts. *m*- and *p*-tolunitriles could be given in doses of 1 g. without toxic effects, but the maximum safe dose of *o*-tolunitrile was 0·7 g. Benzonitrile was more toxic and doses of 0·7 g. caused in some animals considerable toxic symptoms, including anorexia, serous discharge from the eyes, and, in some cases, the excretion of large amounts of reducing material, starting 3–4 days after dosage. Smith & Williams (1950), who gave benzonitrile in olive oil, did not record these effects. It is possible that the toxicity was diminished by delaying absorption, although in a few experiments in which we administered benzonitrile in olive oil we observed definite toxic effects in some rabbits, a dose of 1 g. in olive oil proving fatal to one rabbit. Many rabbits survived a 1 g. dose in water. *p*-Cyanobenzoic, *p*-cyanohippuric, terephthalamic and phthalamic acids caused no obvious toxic effects in doses of 1 g.

Methods of analysis

Ether-soluble acid, ester and ether type glucuronides and ethereal sulphate were estimated as previously described (see Bray *et al.* 1947; Bray, Thorpe & White, 1950*b*). The paper-chromatographic technique was essentially that described in an earlier paper (Bray, Thorpe & White, 1950*a*).

RESULTS

Normal excretion of metabolites

The average daily excretion of ethereal sulphate by the rabbits used in this investigation ranged from 28 to 35 mg. SO_3. The average by which the normal individual daily values differed from the corresponding weekly averages used as 'baseline' for purposes of calculation was ± 8 % (± 3 mg. SO_3). The average daily excretion by individual rabbits of reducing material ranged from 235 to 354 mg. (calculated as glucuronic acid). The average by which the daily individual normal values differed from the weekly averages was ± 6 %. The corresponding values for ether-soluble acid were 623–855 mg. (calculated as hippuric acid) and ± 4 %.

Quantitative experiments with tolunitriles

The results obtained are summarized in Table 1. *m*- And *p*-tolunitriles are excreted mainly as ether-soluble acid, the nature of which is discussed in a later section. The increase in excretion of ester glucuronide with both these isomers and of ethereal sulphate with *m*-tolunitrile is of doubtful significance since the actual increases observed were of the same order as normal baseline fluctuations. About half of the *o*-tolunitrile administered was accounted for by the quantitative results. The only acidic metabolite isolated was phthalic acid. Phthalide, equivalent to about 20 % of the dose, was, however, isolated from hydrolysed *o*-tolunitrile urine, and since phthalide is readily formed from *o*-hydroxymethylbenzoic acid in acid solution, some of the latter compound may also have been present. (We have isolated *o*-hydroxymethylbenzoic acid from acidified urines after giving either phthalide or *o*-hydroxymethylbenzoic acid.) The values obtained for ester glucuronide excretion from *o*-tolunitrile are not regarded as very reliable since the administration of this compound sometimes caused the excretion of large amounts of reducing material. The smaller amounts found here may be due to this rather than to the elimination of an actual metabolite. The phenols responsible for the increased excretion of ethereal sulphate have not yet been identified.

Table 1. *Excretion by the rabbit of metabolites of tolunitriles, expressed as percentage of the dose*

Metabolite	No. of experiments	Range	Average
	o-Tolunitrile		
Ether-soluble acid	7*	5–30	21
Ester glucuronide	7*	0–5	2
Ether glucuronide	4†	8–18	14
Ethereal sulphate	3*	12	12
	m-Tolunitrile		
Ether-soluble acid	7*	78–109	92
Ester glucuronide	7*	0–5	3
Ether glucuronide	3*	0	0
Ethereal sulphate	3*	0–2	1
	p-Tolunitrile		
Ether-soluble acid	16	62–113	86
Ester glucuronide	16	0–7	2
Ether glucuronide	11*	0	0
Ethereal sulphate	3	0	0

* One experiment in this group was made on the pooled urines of ten rabbits.

† Determination by naphthoresorcinol method (Hanson, Mills & Williams, 1944) gave on two other urines values of 22 and 29%.

Qualitative experiments

o-Tolunitrile. The following is typical of several experiments made with urines from rabbits dosed with *o*-tolunitrile. Continuous extraction with ether of *o*-tolunitrile urine as collected (pH 7·8–8·0) gave only urea. Extraction of the residual urine, after acidification with HCl to pH 2–3, gave a mobile brown syrup which on chilling partially crystallized. Trituration with ethanol (96%) yielded a yellow powder which after recrystallization from the same solvent gave colourless crystals, m.p. 192° (with sublimation). On fusion with resorcinol and conc. H_2SO_4 these gave a product which in alkaline solution showed the characteristic fluorescence of fluorescein. The identity of this compound with phthalic acid was confirmed by comparison (mixed m.p.) with an authentic sample. (Found: C, 57·6; H, 3·2. Calc. for $C_8H_6O_4$: C, 57·8; H, 3·6%.) Yield 280 mg. from the 24 hr. urine of ten rabbits, each of which had received 0·7 g. *o*-tolunitrile, i.e. 3% of the dose. The material soluble in ethanol (above) consisted almost entirely of benzoic acid (150 mg.). The urine remaining from the last ether extraction was hydrolysed by boiling for 1 hr. with conc. HCl (0·2 vol.). Extraction with ether then gave a syrupy product from which phthalide, m.p. 70°, identical (mixed m.p.) with an authentic sample, was isolated. Yield 0·7 g., i.e. 21% of dose. The mother liquors from the purification of phthalide yielded a further small amount of phthalic acid (50 mg.). In other experiments phthalide was obtained from extracts of acidified urine without hydrolysis.

As described below, phthalamic acid gives rise in the rabbit to phthalic anhydride, and since the former may theoretically be an intermediate in the conversion of *o*-tolunitrile to phthalic acid, the ether-soluble material extracted from *o*-tolunitrile urine as collected was examined for the presence of phthalic anhydride. (Phthalic acid is not extracted under these conditions.) Although this could not be isolated, the extract gave a strongly positive fluorescein test. An extract of normal rabbit urine obtained under the same conditions gives only a very feeble fluorescence, if any. It is, therefore, probable that a small amount of phthalic anhydride was present.

The quantitative results in Table 1 show that *o*-tolunitrile causes a comparatively large increase in ethereal sulphate and ether glucuronide excretion, but examination of ether extracts of acidified and hydrolysed urines failed to yield any phenolic metabolite. Paper chromatograms (see Bray *et al.* 1950*a*; Bray, Lake, Thorpe & White, 1950) also failed to reveal any phenols other than those known to be present in normal urine. As we have not yet found a solvent mixture suitable for use with very fast moving phenols (e.g. phenol, cresols, xylenols) the metabolite in question may belong to this class. Another possibility is that *o*-tolunitrile increases the output of normal urinary phenols. Preliminary evidence of such an increase was obtained after administration of *o*-toluamide to rabbits (unpublished results), when examination of the urine by paper chromatography suggested the presence of significantly increased amounts of *m*- and *p*-hydroxybenzoic and *p*-hydroxyphenylacetic acids. An attempt to isolate the glucuronide by the usual procedure via the Pb salt (cf. Bray *et al.* 1947) gave a syrup which was partly soluble in ether. The ether-soluble part, which could not be crystallized, was hydrolysed by boiling with HCl (5 N for 1 hr.). Only phenols occurring in normal urine could be detected on paper chromatograms of the ether extract of the hydrolysate and a very small amount of *p*-hydroxybenzoic acid was isolated. Examination of the hydrolysate of the ether-insoluble part showed the presence of *m*- and *p*-hydroxybenzoic acid and also three spots on the paper chromatogram which were not matched by any from normal urine. Two of these had R_F 1·0 in benzene-formic acid, and gave green and orange colours with diazotized *p*-nitraniline; the third spot had R_F about 0·05 (4 hr.) and gave a pale-blue colour. None of these has been identified.

Phthalamic acid. The 24 hr. urine of five rabbits which had each received 1 g. phthalamic acid was extracted at pH 8·3 and from the extract phthalic anhydride (m.p. 130°) was isolated. Yield 300 mg. (7% of the dose). The urine was then acidified (pH 2–3) and re-extracted to give a crystalline extract from which phthalic acid (3·4 g., m.p. 191°) was obtained. Yield, 68% of the dose. Control experiments showed that phthalic anhydride, suspended in urine pH 2–3 for 24 hr. and then continuously extracted with ether, yielded

phthalic acid in amounts indicating considerable conversion. No appreciable amount of phthalic acid was formed if phthalic anhydride was left to stand in urine at pH 7–8. It seems clear, therefore, that any phthalic anhydride isolated can be regarded as a metabolite whereas phthalic acid may be, wholly or in part, an artifact. Phthalamic acid forms no phthalic anhydride or phthalic acid on standing in urine at pH 7–8, but forms phthalic acid at pH 2–3.

o-Hydroxymethylbenzoic acid and phthalide. Examination of the urine of rabbits which had received either of these compounds failed to reveal the presence of either phthalic anhydride or phthalic acid. Only phthalide or *o*-hydroxymethylbenzoic acid could be isolated.

m-Tolunitrile. The urine of rabbits dosed with this compound was examined by methods similar to those described below for *p*-tolunitrile urine. From the ether-soluble acid fraction colourless needles, m.p. 217°, were obtained and identified as *m*-cyanobenzoic acid. The mixed m.p. with an authentic sample (m.p. 217°) was 217°. (Found: N, 9·9. Calc. for $C_8H_5O_2N$: N, 9·5 %.) Average yield, 61 % of the dose. No *m*-cyanohippuric, *iso*phthalamic or *iso*phthalic acid could be detected.

m-Cyanobenzoic acid. 1 g. of this acid was given to each of ten rabbits. The pooled 24 hr. urine was acidified (pH 3) and exhaustively extracted with ether. Only *m*-cyanobenzoic acid could be isolated from the extract. Yield, 6·6 g. (66 % of dose).

p-Tolunitrile. Several large-scale experiments were carried out with urine from rabbits dosed with *p*-tolunitrile. While the results obtained for the major metabolite were consistent, the amounts of the minor metabolites isolated varied considerably. (1) The ether-soluble acid obtained by continuous extraction with ether of *p*-tolunitrile urine at pH 2–3 was fractionated by refluxing with several successive portions of toluene (50 ml. each). The soluble material was crystallized from water giving colourless leaflets m.p. 216°, mixed m.p. with authentic *p*-cyanobenzoic acid (m.p. 219°) 219°. (Found: N, 9·7; equiv. (by titration) 147. Calc. for $C_8H_5O_2N$: N, 9·5 %; equiv. 147.) Yield 48–60 % of the dose. The material insoluble in toluene was a brown tar. This was dissolved in hot water and purified with charcoal. In one experiment, the cooled solution deposited 50 mg. (from 4 g. *p*-tolunitrile) of crystals which did not melt, but sublimed at 250–300°. These were shown to be terephthalamic acid by comparison with an authentic specimen and by conversion to the methyl ester (m.p. 201–203°). (Found: N, 8·5. Calc. for $C_9H_7O_3N$: N, 8·5 %.) It is unlikely that terephthalamic acid was an artifact since none was formed when added *p*-cyanobenzoic acid was extracted from acidified normal urine and then treated by the toluene fractionation procedure as above. (2) In another experiment the mother liquors from the recrystallization of *p*-cyanobenzoic acid deposited shining yellow plates, m.p. 190°, shown by comparison (mixed m.p.) with an authentic sample to be *p*-cyanohippuric acid. (Found: N, 13·3. Calc. for $C_{16}H_8O_3N_2$: N, 13·7 %.) A further small amount was obtained from the fraction of the ether-soluble acid insoluble in toluene. Total yield 700 mg. from 4 g. *p*-tolunitrile (10 % of the dose). (3) In another experiment all three metabolites were isolated. The yields were 6·6 g. *p*-cyanobenzoic acid, 20 mg. *p*-cyanohippuric acid, and 50 mg. terephthalamic acid from 8·8 g. *p*-tolunitrile, i.e. 60, 0·13 and 0·4 %, respectively, of the dose. The urine after acid extraction was hydrolysed by boiling for 1 hr. with conc. HCl (0·2 vol.) and again extracted with ether. Treatment of the crystalline extract with boiling water left an insoluble residue (100 mg.) which did not melt, but sublimed at 250–300°. It contained no N and was shown to be terephthalic acid by comparison with an authentic specimen and conversion to its dimethyl ester, m.p. 140°, mixed m.p. with authentic sample (m.p. 141°) 140°. It is probable that the terephthalic acid is an artifact formed from unextracted terephthalamic acid so that the total yield of the latter could be assessed as 1·2 % of the dose. The amount (10 % of the dose) of *p*-cyanohippuric acid isolated in the second experiment compared to that (0·0, 0·13 %) obtained in the other experiments requires comment. It may be relevant that in the second experiment the animals were dosed under fasting conditions before receiving their daily food ration, whereas in the others they were dosed after having consumed some food.

p-Cyanobenzoic acid. 1 g. of this compound was administered to each of five rabbits. The pooled 24 hr. urine was acidified (pH 3) and continuously extracted with ether. The extract on purification yielded only *p*-cyanobenzoic and *p*-cyanohippuric acids in amounts corresponding to 50 and 2 %, respectively, of the dose.

p-Cyanohippuric acid. This was administered to rabbits in doses of 1 g. and the acidified urines treated as in the previous section. Only *p*-cyanobenzoic acid (yield 50 % of dose) was isolated, suggesting that the glycine conjugate is hydrolysed in the gut. No glycine conjugate was detected in the ether-soluble acid.

Terephthalamic acid. The ether-soluble acid isolated from acidified terephthalamic acid urine by continuous extraction for a week was triturated with hot ethanol to remove tarry material. The white residue contained N and after further purification was shown to be terephthalamic acid. Yield 2·9 g. (58 % of the dose). No terephthalic acid could be detected.

The yields of the compounds isolated from urine after administration of the tolunitriles and related compounds are summarized in Table 2. The values given are those for the highest yields obtained.

Table 2. *Metabolites isolated from urine after administration of tolunitriles and related compounds, expressed as percentage of dose*

Compound administered	Cyanobenzoic acid	Cyanohippuric acid	Carbamyl benzoic acid	Benzene dicarboxylic acid	Phthalic anhydride	Phthalide
o-Tolunitrile	0	0	0	3·5	Trace	21
Phthalamic acid	—	—	—	68	7	—
m-Tolunitrile	61	0	0	0	—	—
m-Cyanobenzoic acid	66	0	0	0	—	—
p-Tolunitrile	60	10	0·9	0·8	—	—
p-Cyanobenzoic acid	50	2	0	0	—	—
p-Cyanohippuric acid	50	0	0	0	—	—
Terephthalamic acid	—	—	58	0	—	—

Metabolism of benzonitrile

Analyses were carried out on benzonitrile urines as described for the tolunitriles. Benzonitrile is, however, an extremely toxic compound, and animals receiving it did not consume the whole of their food ration for several days after dosage. The baseline excretion values of the metabolites estimated are, therefore, likely to be unreliable. The excretion of ethereal sulphate was definitely increased and the percentages of the dose apparently excreted in this form were found to be 20–21 %. It is likely that these results are low. The metabolism of benzonitrile appears to be a slow process, since the increase in ethereal sulphate excretion persisted for 2 days after dosage. The ether-soluble acid results were too variable to justify any conclusion drawn from them. As already mentioned, benzonitrile caused in some animals an enormous increase in the excretion of reducing material which was usually not apparent until 3 or 4 days after dosage and which lasted up to 3 or 4 further days. During this period as much as 7–8 g. extra reducing material (calculated as glucuronic acid) was excreted, a considerable part of which was shown to be due to glucuronic acid by the quantitative naphthoresorcinol method of Hanson, Mills & Williams (1944). Thus the reducing values of benzonitrile urine cannot be used as a reliable indication of the metabolic fate of the compound.

In view of Smith & Williams's (1950) more detailed study our qualitative investigation was confined to examination of extracts of benzonitrile urine by paper chromatography. Using a mixture of *n*-butanol (4 parts), pyridine (8 parts), saturated aqueous NaCl (5 parts) and NH_3 (sp.gr. 0·880, 1 part) as developing solvent and diazotized *p*-nitraniline as detecting reagent, *o*- and *m*-cyanophenols (R_F 0·8 and 0·9, respectively, after 15 hr.) were detected in extracts of hydrolysed benzonitrile urine. *m*-Cyanophenol was also detected using a mixture of light petroleum (b.p. 60–80°) (5 parts), water (1 part) and glacial acetic acid (5 parts) as solvent. (R_F 0·1 after 5 hr.) It is likely that the *p*-isomer was also present, but it was not detected since it gives no colour with any of the spraying reagents used. The results of Smith & Williams (1950) suggest that only *m*- and *p*-cyanophenols are excreted as ethereal sulphates, so that the *o*-isomer probably forms only a glucuronide. The chromatograms suggested that the amount of *o*-cyanophenol present was of the same order as that of *m*-cyanophenol.

DISCUSSION

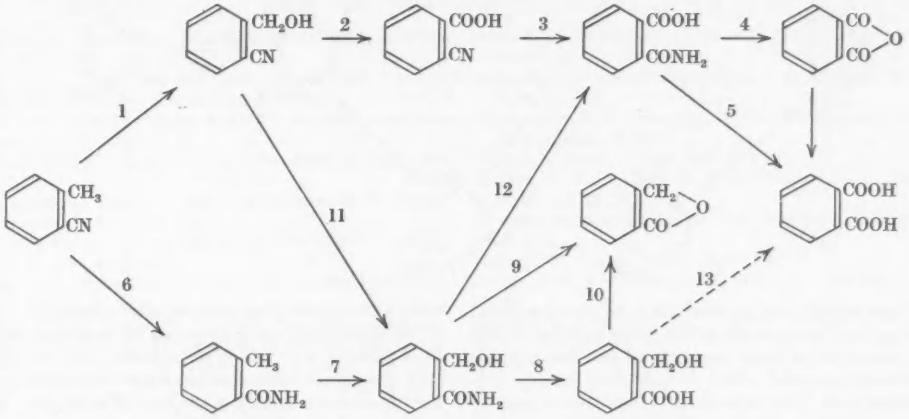
From the foregoing results it is clear that the nitrile group may be hydrolysed in the rabbit although with difficulty in some compounds. Smith & Williams (1950), on the basis of the isolation of 500 mg. benzoic acid from urine, claimed that about 10 % of benzonitrile is hydrolysed. It is doubtful, however, whether the isolation of benzoic acid under these circumstances proves that it is a metabolite of benzonitrile. It is unlikely that 500 mg. of benzoic acid would have been isolated from 10 days' normal rabbit urine, although such an amount could be present, since isolation procedures from urine are usually far from efficient. We have, however, isolated comparably large amounts of benzoic acid from the urine of rabbits which had received 3:5-xylen-1-ol, *o*-toluamide, *o*-tolunitrile and 2-methylbenzoxazole. Benzoic acid would not be expected as a metabolite of any of these. Smith & Williams (1950) also reported the isolation of benzoylglucuronide from benzonitrile urine. If benzoic acid had been formed to such an extent that benzoylglucuronide was excreted in sufficient quantity to be isolated, it would have been expected that an even greater amount of hippuric acid would also have been formed, since it is well known that in the rabbit the conjugation of benzoic acid with glycine takes place to a much greater extent than conjugation with glucuronic acid. Smith & Williams (1950) did not comment upon the amount of hippuric acid isolated. It seems, therefore, reasonable to attribute their findings to the inhibition of glycine conjugation by the administration of benzonitrile. The formation of cyanophenols from benzonitrile in the amounts which they claimed indicates that hydroxylation takes place more readily than does hydrolysis of the nitrile group.

From the behaviour of the tolunitriles it is clear that modification of the nitrile group does occur, although only to a small extent with the *p*-isomer and possibly not at all with the *m*-isomer. In these two compounds the methyl group is more readily oxidized. In view of the isolation of phthalide and phthalic acid from *o*-tolunitrile urine and of terephthalamic and terephthalic acid from *p*-tolunitrile urine, we must modify our earlier opinion (Bray, Humphris & Thorpe, 1949; Thorpe, 1950) that in compounds containing two potential centres for conjugation only one is modified in a single molecule. Both potential centres must have been changed, in order to form the benzenedicarboxylic acid derivatives which were isolated as metabolites.

The only metabolites of *o*-tolunitrile isolated were phthalide and phthalic acid. No evidence was obtained of the excretion of *o*-cyanobenzoic or *o*-toluic acids. The available information relevant to the fate *o*-tolunitrile in the rabbit can be summarized as follows: (*a*) *o*-Hydroxymethylbenzoic acid (or phthalide) is known to be a metabolite of *o*-toluamide. It is probable that *o*-hydroxymethylbenzamide is an intermediate in this reaction (Bray, Thorpe & Wood, 1949). (*b*) Ether extracts of *o*-toluamide urines only give a very feeble fluorescein test so that at the most only minute amounts of phthalic anhydride (or acid) are formed in the rabbit from *o*-toluamide. (*c*) When phthalide or *o*-hydroxymethylbenzoic acid is given to a rabbit no phthalic anhydride or phthalic acid can be detected. (*d*) *o*-Toluic acid gives rise in the rabbit to neither phthalide (Bray, Thorpe & Wood, 1949), nor phthalic anhydride (or acid). (*e*) Only phthalic acid and its anhydride were isolated from the urine when phthalamic acid was given to the rabbit. (*f*) *o*-Hydroxymethylbenzoic acid is readily converted to

phthalide in acid solution. The fact that phthalide is often isolated in better yield after the urine has been treated with acid (standing at pH 2·3 or under hydrolysis conditions) favours the view that *o*-hydroxymethylbenzoic acid may be the primary metabolite, but there is as yet no conclusive evidence. (*g*) Phthalic acid may be an artifact formed from phthalamic acid or from phthalic anhydride owing to acid treatment of the urine.

The scheme below has been drawn up, taking these facts into consideration and provides an explanation for the observed metabolites and artifacts. It cannot at present be decided whether phthalide is formed via reactions 1 and 11 or 6 and 7, since we have so far failed to synthesize *o*-hydroxymethylbenzonitrile and *o*-hydroxymethylbenzamide. It seems probable that the formation of phthalic acid proceeds via *o*-cyanobenzoic acid (reactions 1, 2 and 3) although none of the intermediates has actually been isolated. In view of its lability, isolation of *o*-cyanobenzoic acid could hardly be expected. Phthalic acid may be regarded as an artifact derived from either phthalamic acid or phthalic anhydride. Phthalamic acid is readily converted to phthalic anhydride (or acid) by the rabbit. The alternative route via reactions 1, 11 (or 6, 7) and 12 is improbable since only traces of phthalic acid are formed from *o*-toluamide. An argument against the route 1, 11 (or 6, 7), 8 and 13 is that *o*-hydroxymethylbenzoic acid does not give rise to phthalic acid. It must, however, be admitted that there is no direct evidence for the carbamyl intermediates in the scheme.



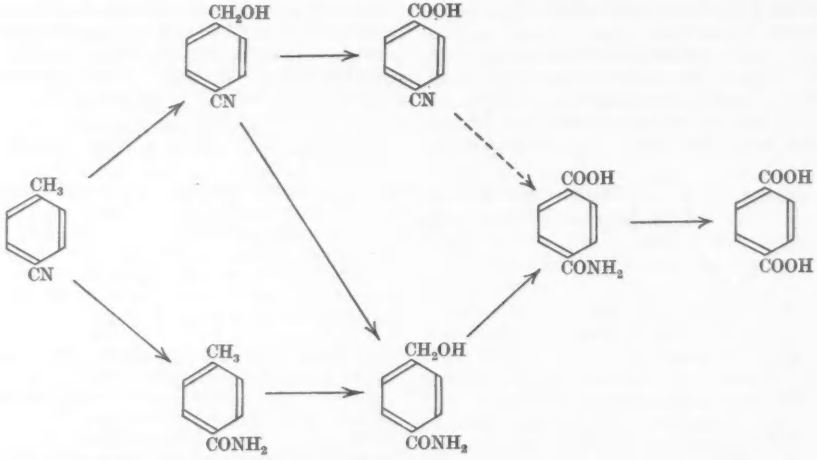
The only metabolite of *m*-tolunitrile identified was *m*-cyanobenzoic acid. This was isolated in such large amount that it is reasonable to assume that ether-soluble acid excreted (92% of dose) was mainly composed of this compound, since no glycine conjugation could be detected.

The terephthalic acid isolated from hydrolysed *p*-tolunitrile urine was almost certainly an artifact. Terephthalamic acid appears to be a true metabolite of *p*-tolunitrile. This acid was also identified as a metabolite of *p*-toluamide (Bray, Thorpe & Wood, 1949). Terephthalamic acid might be formed from *p*-tolunitrile either via *p*-cyanobenzoic acid or via *p*-hydroxymethylbenzamide as shown in the scheme (p. 198). Since we failed to detect any terephthalamic acid as a metabolite of *p*-cyanobenzoic acid, it seems probable that the small amount of terephthalamic acid produced from *p*-tolunitrile has been formed either via *p*-toluamide or via *p*-hydroxymethylbenzonitrile. The main metabolic reaction is the formation of *p*-cyanobenzoic acid which appears to be readily eliminated either as such or conjugated with glycine.

The study of the tolunitriles has shown that the stability of the nitrile group is considerably affected by the position of the methyl group. In the ortho compound at least 23% is hydrolysed to carboxyl, in the para more than 1%, but in the meta either none, or so little as to prevent isolation of the metabolites. There is no doubt that the methyl group is oxidized readily in the meta and para isomers, but whether the apparent absence of any hydrolysis of the nitrile group in the meta compound is due to the formation or excretion of *m*-cyanobenzoic acid with exceptional ease, leaving little opportunity for the action of the enzyme system concerned with hydrolysis of the nitrile group, must be left for further study. The position with regard to the *o*-tolunitrile is as unsatisfactory as that which we found with *o*-toluamide (Bray, Thorpe & Wood, 1949) in that not more than one half of the dose has been accounted for. The substance (or substances) responsible for the increased excretion of ethereal sulphate and ether glucuronide has not been identified. There is no

support for the idea that hydrogen cyanide is liberated leaving *o*-cresol (cf. Adeline *et al.* 1926). Had the increased elimination of ethereal sulphate been due mainly to *o*-cresol, a larger excretion of ether glucuronide would have been expected (cf. Bray *et al.* 1950*b*).

certain, however, whether the role of the enzyme *in vivo* is hydrolytic or synthetic, or whether the synthesis of glucuronic acid is brought about by the same process as that responsible for the formation of glucuronides. Deichmann, Kitzmiller & Witherup (1945) have observed that the ability of rabbits to



Benzonitrile and *o*-tolunitrile may now be added to the list of compounds which cause in some rabbits the excretion of large amounts of non-fermentable reducing material which is probably mainly glucuronic acid. The other compounds are: *o*- and *m*-acetotoluidides (Bray & Thorpe, 1948), *o*-xylene (Bray, Humphris & Thorpe, 1949), *o*-toluamide (Bray, Thorpe & Wood, 1949), *o*-aminophenol, benzoxazolone (unpublished results). Smith & Williams (1949*a*, *b*) have made similar observations with aniline, *p*-phenetidine and the anisidines. They suggest that the reducing material, which they showed to be glucuronic acid, resulted from the breakdown of labile glucuronides, the nature of which could not be elucidated. In some of our experiments, however, the amount of reducing material was much too great to be accounted for by the dose administered and its excretion, which was not an invariable response, often persisted for periods of several days after the dose, whereas glucuronides formed as a result of conjugation of administered foreign compounds are usually excreted rapidly. It is of interest that Levvy, Kerr & Campbell (1948) have observed an increase in the glucuronidase activity *in vitro* of the livers and kidneys of mice which had been treated with menthol, carbon tetrachloride, chloroform, mercuric nitrate, chloroform, uranyl acetate, yellow phosphorus, or phenylarsenoxide, and suggest that it is a consequence of cell proliferation following tissue damage. It is not yet

form and excrete glucuronides after administration of *cyclo*hexanone is not impaired by treatment with liver poisons, e.g. yellow phosphorus, and conclude that glucuronic acid excretion is not an indication of the functional integrity of the liver. The origin of the reducing material observed in our experiments, which certainly cannot be entirely derived from conjugates of metabolites of the compounds administered, is still obscure. Experimental study is rendered difficult by the uncertain response of the animals, but it is hoped to investigate this problem further.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-tolunitriles and benzonitrile in the rabbit has been studied.

2. *o*-Tolunitrile gives rise to phthalide and phthalic acid (21 and 3 % of the dose, respectively, by isolation) and also increases the excretion of ethereal sulphate by an amount equivalent to 12 % of the dose.

3. *m*- And *p*-tolunitriles are excreted almost entirely as ether-soluble acid (92 and 86 % of the dose, respectively). *p*-Cyanobenzoic and *p*-cyanohippuric acids (60 and 10 % of the dose, respectively) were isolated from *p*-tolunitrile urine. A small amount of terephthalamic acid (1·2 % of the dose) was also isolated. The only metabolite of *m*-tolunitrile detected was *m*-cyanobenzoic acid (61 % of the dose was isolated).

4. At least 20–21 % of a dose of benzonitrile is excreted as ethereal sulphate. *o*- And *m*-cyanophenols were detected by paper chromatography in ether extracts of hydrolysed benzonitrile urine. In some experiments the administration of benzonitrile gave rise to the excretion of very large amounts of reducing material.

5. Qualitative examination of the excretion products obtained after administration of some possible metabolites of the tolunitriles has been made. *m*-Cyanobenzoic acid was excreted unchanged (66 % of dose). No glycine conjugation could be detected. *p*-Cyanobenzoic acid was excreted mainly unchanged, but was conjugated to a small extent with glycine (50 and 2 % of the dose isolated, respectively). *p*-Cyanohippuric acid was excreted mainly as *p*-cyanobenzoic acid (50 % of the dose isolated); no glycine conjugate was detected. Phthalic anhydride and phthalic acid (7 and 86 % of the dose, respectively) were isolated after administration of phthalamic acid. Only unchanged terephthalamic acid (58 % of the dose) could be isolated after giving terephthalamic acid.

The micro-analyses were carried out by Drs Weiler and Strauss, Oxford, and by Miss B. G. Humphris.

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Biochemistry of Locusts

5. THE GREEN PIGMENT OF THE HAEMOLYMPH AND INTEGUMENT OF SOLITARY LOCUSTS (*LOCUSTA MIGRATORIA MIGRATORIOIDES*, R. & F., AND *SCHISTOCERCA GREGARIA*, FORSK.)

By T. W. GOODWIN and S. SRISUKH
Department of Biochemistry, The University of Liverpool

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In the solitary phase locusts are often green; this is not an essential characteristic of solitary insects because other colours, such as buff, are often encountered, but it is probably true to say that the green pigment does not occur in the integument of gregarious insects. Similarly, the haemolymph from solitary locusts is almost invariably bright green whilst that from gregarious locusts is golden-yellow; a light-green haemolymph is, however, very occasionally encountered in the latter. The present paper reports an investigation into the green pigment.

Green pigments are widespread in insects, but not a great deal is known about them. As early as 1882, Krukenberg considered that the green pigment of *Locusta* (*Tettigonia*) *viridissima* was different from chlorophyll although, later, Podiapolsky (1907) stated that the absorption spectrum of the pigment was somewhat similar to that of chlorophyll. Rearing experiments on a number of insect species showed, however, that the formation of the green pigment was independent of the amount of chlorophyll in the diet (Toumanoff, 1927; Giersberg, 1928); in particular, Faure (1932) noted that the produc-

tion of the green pigment in locusts depended on succulent green grass, but that the pigment was not chlorophyll. The first suggestion that the green pigments of insects, which in different species appear to be very similar, were heterogeneous was put forward by Przibram & Lederer (1933) who separated the green pigment of the haemolymph of *Dixippus morosus* into blue and yellow components; this has recently been confirmed by Junge (1941) and Okay (1945) in a number of insect species. Junge (1941), who gave the name 'insectoverdin' to the green complex, found that all the pigments he examined consisted of two chromoproteins, the prosthetic group of the blue component being a bile pigment and that of the yellow component either β-carotene or lutein. He considered the bile pigment to be an isomer of biliverdin and called it glaucobilin; Lemberg & Legge (1949) consider that this pigment is mesobiliverdin. Okay (1945), examining *Mantis religiosa* and many other Orthoptera, came to similar conclusions, but in some cases considered the yellow component to be a yellow water-soluble non-protein compound. He termed the bile pigment orthopterocyanobilin, which Lemberg & Legge (1949) suggest may be identical with mesobiliviolin.

Having reared solitary locusts (*Schistocerca*) to the fifth instar on a chlorophyll-free diet, Chauvin (1939) decided that the green pigmentation that he observed was either a relic of the chlorophyll present in the egg or a pigment synthesized *de novo*. Later, Chauvin (1941) considered that the green coloration was due to flavone (yellow) (responsible for the yellow coloration of adult gregarious *Schistocerca*) being so distributed on the chitin as to appear green. It has now been shown that the yellow coloration of gregarious *Schistocerca* is due to β-carotene and not to a flavone (Goodwin & Srisukh, 1950*a*).

EXPERIMENTAL

Collection and examination of haemolymph. The locusts were obtained from the Anti-Locust Research Centre where, in order to obtain them in phase *solitaria*, each was reared separately in a 1 l. cage. When received in this laboratory they were kept in a warm room with plenty of food (succulent grass) for some hours before the haemolymph was collected. Collection was carried out by piercing with a pin a small hole in the thin region of the integument just above the connexion between the hind-leg and the integument and then squeezing the insect gently but firmly until the lymph (about 2–3 drops/insect) dripped into a small test tube containing either distilled water or a phosphate buffer of pH 7; the insects quickly recover from this treatment. Invariably during the collection a small amount of bright-yellow coagulum was formed on the side of the test tube; the diluted haemolymph was either yellow or green.

The coagulum and solution were examined in the usual way for carotenoproteins (Goodwin & Srisukh, 1949); the solution was also examined for bile pigments by adding an equal volume of ethanol, removing the carotenoids by extraction with light petroleum, acidifying with HCl, and extracting with ethyl acetate. Insectorubin was then tested for in the aqueous phase (Goodwin & Srisukh, 1950*b*).

The green pigment of the integument. The insects were killed, the fat bodies removed and the green portions of the integument separated from the rest. The green pigment was extracted from the tissues by grinding with sand and a very small volume of water. The mixture was then centrifuged and the residue, which contained free carotenoids and insectorubin, was discarded. The supernatant liquid was examined as follows: addition of $(NH_4)_2SO_4$ to half saturation yielded a brown precipitate which was filtered off and discarded. If the filtrate was found to contain combined carotenoids these were removed and then one-tenth the volume of conc. HCl was added and the solution (*A*) exhaustively extracted with ethyl acetate. The ethyl acetate was washed with a little water and then distilled off *in vacuo*; the residue was a small volume of a deep-blue aqueous-HCl solution from which the pigment only slightly soluble in pure ether was extracted with a mixture (1:1) of ether and methanol. The pigment was then purified by chromatography on acid-washed talc (Goodwin & Srisukh, 1950*b*); it was strongly adsorbed near the top of the column as a bright-blue zone and elution with water followed by methanol removed traces of a yellow impurity; the purified pigment was then eluted with methanol containing 5% (v/v) of conc. HCl and its properties examined in as much detail as was possible with the very small amounts available. The residual aqueous solution (*B*) was concentrated *in vacuo* and the resulting material chromatographed on talc. A very small bluish-grey zone was obtained, obviously due to traces of the pigment just discussed, but elution with water separated a very small quantity of a thermostable pigment.

Most of the experiments were carried out on solitary *Locusta*, but a few experiments with *Schistocerca* gave identical results.

RESULTS

Haemolymph. The absorption spectra of typical samples of haemolymph from solitary and gregarious locusts are recorded in Fig. 1. It will be seen that they differ quite strikingly in that the green *solitaria* haemolymph exhibits a very sharp band at 673 mμ., which almost completely disappears on standing. On standing overnight haemolymph from both phases darkens, that from *solitaria* being slightly bluer than that from *gregaria*. This darkening is almost certainly due to the action of tyrosinase for such an action has previously been noted by Dennell (1947) in isolated haemolymph of a number of insect species. The slight blue appearance assumed by aged *solitaria* haemolymph may be due to the presence of small amounts of blue pigment, although most appears to be destroyed. In both phases the haemolymph contains considerable amounts of a carotenoid attached to a protein, but no free carotenoids. The carotenoid was identified as β-carotene in both cases; no astaxanthin was detected. Most of the β-carotene occurred in the yellow coagulum which was always formed when lymph was being collected.

The *gregaria* haemolymph proteins yielded no insectorubin or blue pigment, whilst the solitary

haemolymph contained a blue pigment but not insectorubin. This was obtained only in minute amounts, but appeared to be identical with the blue pigment extracted from the integument.

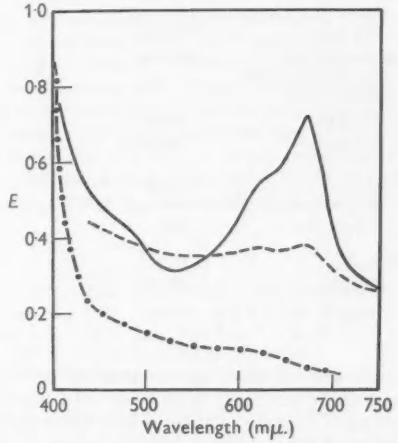

Fig. 1. The absorption spectrum of locust haemolymph. ——, haemolymph from solitary locusts—fresh; – – – –, haemolymph from solitary locusts—after standing 24 hr.; –·–·–·–, haemolymph from gregarious locusts.

The green pigment complex of the integument of solitary locusts. This pigment, which is water-soluble and shows an absorption spectrum ($\lambda_{max.}$ 673 mμ.) very similar to that of the green *solitaria* haemolymph (see Fig. 1), can be separated into two major fractions: (1) yellow, soluble in ether; and (2) blue, soluble in ethyl acetate; a third and minor yellow fraction soluble in water also exists.

The ether-soluble fraction contained a mixture of two carotenoids, attached to protein. After chromatographic separation of these pigments they were identified as β-carotene and free (unesterified) astaxanthin by their chromatographic, spectrographic and partition behaviour.

After chromatographic purification, the blue ethyl acetate-soluble pigment exhibited, in different solvents, the spectra recorded in Fig. 2. When dissolved in methanol and treated with methanolic zinc acetate the solution became green and exhibited a spectrum with maxima at 625 and 685 mμ. (Fig. 3); addition of iodine alters the spectrum considerably and only one band (at 632 mμ.) is observed (Fig. 3). These and other properties recorded in Table 1 make it extremely likely that the blue pigment is mesobiliverdin. The properties of authentic mesobiliverdin are also shown in Table 1 for comparison.

The water-soluble material occurs in amounts much too small for full study. It is heat-stable, does not occur as a protein complex, it fluoresces bluish-purple in ultraviolet light, retains its fluorescence in 0·1 N-sodium hydroxide and glacial acetic acid and on the addition of sodium dithionite ($Na_2S_2O_4$), and

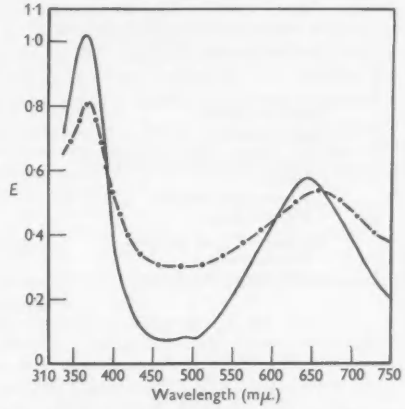

Fig. 2. The absorption spectrum of the bile pigment (probably mesobiliverdin) present in the integument of green solitary locusts as the prosthetic group of a blue chromoprotein. ——, in dioxan; –·–·–·–, in methanol containing 5% (v/v) of conc. HCl.

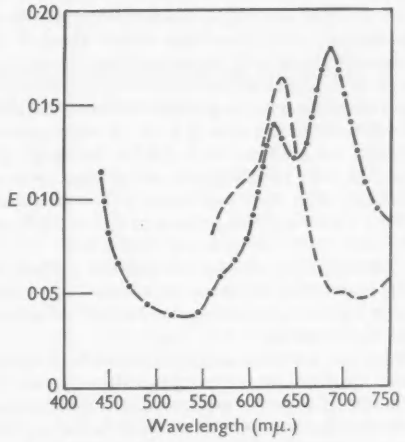

Fig. 3. The absorption spectrum of the zinc acetate bile pigment complex. –·–·–·–, before the addition of I_2; – – –, after the addition of I_2.

exhibits an absorption spectrum with a band at 282 mμ. It appears to be very similar to the pterin-like pigment present in the eyes of locusts (Goodwin & Srisukh, 1951).

Table 1. *A comparison of the properties of the blue pigment obtained from green locusts and of mesobiliverdin*

Property	Locust pigment	Mesobiliverdin*
Absorption maxima in methanol containing 5% (v/v) conc. HCl	367, 647 mμ.	363, 670 mμ.
In neutral solvents (ether, dioxan)	636 mμ.	635 mμ.
Stability in conc. H_2SO_4 at 100°	Stable for at least 15 min. then gradually changes to green	Stable
Gmelin reaction	Positive	Positive
Diazo reaction	Negative	Negative
Complex with zinc acetate		
Colour	Green	Green
Absorption maxima	625, 685 mμ.	685 mμ.
Fluorescence	Nil	Nil
Addition of I_2 to complex		
Fluorescence	Pink	Red
Absorption maximum	632 mμ.	630 mμ.

* Lemberg & Legge (1949).

DISCUSSION

The green pigments of the haemolymph and integument of solitary locusts are undoubtedly chromoprotein complexes of the insectoverdin type (Junge, 1941). They differ from each other in one major respect, for although they both have the same blue component (mesobiliverdin-protein complex), the yellow component of the haemolymph yields on denaturation only β-carotene whilst that of the integument yields both β-carotene and free astaxanthin. This indicates either that the integument contains two yellow chromoproteins or that β-carotene and astaxanthin are attached to the same protein. Although no evidence is available to settle this point, the first possibility is, on general grounds, more likely. The small amounts of yellow pterin-like pigment which are also present in the extracts, can make little contribution to the final colour of either the haemolymph or the integument. Chauvin's (1941) claim that the green pigment of the integument is a flavone cannot, on the basis of the present work, be accepted.

The absence of astaxanthin from the haemolymph, but its presence in the integument, seems to offer further proof of the conversion of β-carotene, and not xanthophylls, into astaxanthin in locusts, a fact previously noted in developing locust eggs (Goodwin, 1949). It also appears that the site of conversion in the insect is the integument itself.

The data recorded in Table 1 make it reasonably certain that the bile pigment in green locusts is that noted by Junge (1941) in a number of other insects, namely mesobiliverdin. The only differences in properties between our pigment and Junge's are (*a*) that his appeared to be less stable in concentrated sulphuric acid at 100°, and (*b*) that his could not apparently be extracted from ether with N-hydrochloric acid; in spite of these minor differences, it appears that our pigment is the same as that investigated by Junge.

The only point at which we diverge from the recorded data for mesobiliverdin is the position of the absorption maximum of the long-wave band when the solvent is acid methanol. We find $\lambda_{max.}$ to be at 647 mμ., whereas Pruckner & Stern (1937) record a peak at 670 mμ.

As carotenoproteins are present in both gregarious and solitary insects a characteristic difference between the two phases is thus the production of mesobiliverdin in solitaries. This cannot be accepted as a hard and fast differentiation because very occasionally green haemolymph (but not green integuments) are encountered in gregarious insects and solitaries are not always green. The production of the green pigment is always accompanied by a failure to produce integumental melanin, another characteristic of solitary hoppers (Goodwin, 1950), and it is interesting to note in this connexion that the change in isolated haemolymph of insects to a grey-black colour occurs in both solitary and gregarious haemolymphs. This suggests that solitary hoppers are still potentially capable of producing melanin and that their failure to do so is due rather to an inhibition of tyrosinase than to a complete disappearance of the enzyme.

Insectorubin could never be detected in the haemolymph and this strongly suggests that it is not synthesized in the gut but in the integument from a precursor present in the haemolymph. This observation also provides further proof against Chauvin's (1941) idea that this pigment is absorbed by the insect from its food.

SUMMARY

1. The haemolymph and the green parts of the integument of solitary locusts contain typical insectoverdins which can be resolved into yellow and blue components which are chromoproteins.

2. The prosthetic group of the blue chromoprotein is in each case probably mesobiliverdin. The yellow component of the haemolymph is a β-carotene-protein complex whilst that of the integument contains both β-carotene and free astaxanthin; this probably means the co-existence of two yellow chromoproteins in the integument, although the possibility of both carotenoids being attached to the same protein has not been ruled out.

3. A small amount of a yellow water-soluble pterin-like material also occurs in the haemolymph and integument; its contribution to the green coloration can only be slight.

4. Neither astaxanthin nor insectorubin occurs in haemolymph. Both are therefore probably synthesized in the integument, the former from β-carotene and the latter from unknown precursors.

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Derivatives of Adrenaline and Noradrenaline in an Extract of an Adrenal Medullary Tumour

By T. B. B. CRAWFORD
The Pharmacological Department, University of Edinburgh

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Although pharmacological evidence of the occurrence of noradrenaline in the body has been accumulating since Barger & Dale's (1910) classical work on the sympathomimetic amines (for references, see Bergström, Euler & Hamberg, 1950), the first unequivocal demonstration of its presence in the animal body was made by Holton (1949). Using a paper chromatographic technique devised by James (1948), Holton found that the main pressor component of extracts of adrenal medullary tumours was noradrenaline, adrenaline also being present but in much smaller quantity. This finding has been confirmed by Goldenberg, Faber, Alston & Chargaff (1949), who further showed the presence of noradrenaline in U.S.P. adrenaline prepared from cattle suprarenals. Euler & Hamberg (1949) have also demonstrated chemically the occurrence of noradrenaline, in addition to adrenaline, in extracts of cattle suprarenals. Moreover Bergström *et al.* (1950) and Tullar (1949) have isolated it from this source.

Through the kindness of Prof. G. W. Pickering, of St Mary's Hospital, London, the author had the opportunity of examining an extract of a human adrenal medullary tumour by paper chromatography. Adrenaline, noradrenaline, and a number of related compounds may be detected on the developed chromatogram, by spraying the dried paper with a solution of potassium ferricyanide in phosphate buffer pH 7·8 (James, 1948). With this reagent the compounds yield red or purplish-red derivatives. A chromatogram of the tumour extract, on spraying with the ferricyanide reagent, showed the presence of four substances yielding red-coloured derivatives. Of these, two occupied positions which corresponded

with those of noradrenaline and adrenaline. Further investigation indicated that the other two substances were derivatives of adrenaline and noradrenaline. Isolation of these substances for complete chemical characterization was not possible through lack of material. Evidence was obtained, however, which pointed to the probability of their being esters of the amines formed by the union of the secondary alcoholic group on the side chain with the carboxyl group of lactic acid.

As far as is known, the only report in the literature of such a type of compound being present in biological material has been that of Kendall (1932), who found in an extract of adrenal glands a substance which he considered to be lactyladrenaline. In the more recent investigations on extracts of adrenal glands and adrenal medullary tumours (Holton, 1949; Euler & Hamberg, 1949; Goldenberg *et al.* 1949) in which the presence of adrenaline and noradrenaline was demonstrated by paper chromatography using the ferricyanide reagent, no evidence of any other similarly reacting compound was obtained. In view of this, it was necessary to consider the possibility that the additional compounds detected by the author did not actually occur as such in the tumour and appeared in the final preparation as artifacts of the extraction procedure. Further experiments showed that such was indeed highly probable. The author, however, feels that these findings should be communicated if only for the reason that they demonstrate the danger of the possible formation of artifacts in the preparation of biological materials. In addition, it is not inconceivable that these esters may have some biological significance in view of their relative ease of formation and of breakdown, and also of their relative lack of pharmacological activity when compared with the parent amines.

EXPERIMENTAL

Preparation of extract of adrenal medullary tumour

The adrenal medullary tumour was received in small pieces immersed in 0·1 N-HCl (10 ml./g.). The tissue and the brown fluid were ground together in a glass mortar with a little acid-washed silver sand. The extract was centrifuged and the supernatant adjusted to pH 4 (glass electrode) by the addition of 2 N-NaOH. To remove protein, the solution was poured into 15 vol. aldehyde-free ethanol containing 0·05 ml. conc. HCl (sp.gr. 1·18)/100 ml. After standing at approx. 0° for 2 hr., the solution was freed from precipitated protein by centrifugation and evaporated to dryness *in vacuo* at 60–65°. The reddish-brown residue was taken up in 0·01 N-HCl (1 ml./0·5 g. tumour). Some reddish-brown insoluble material was centrifuged off to give the yellow, faintly opalescent solution which was used in the experiments described below.

Paper chromatograms

All chromatograms were carried out by the capillary ascent method of Williams & Kirby (1948). A sheet of Whatman no. 1 filter paper (37 × 39 cm.) was fashioned into cylindrical form by joining the longer sides edge to edge with cellulose tape. Test solutions were applied as single or replicate drops, each 5 μl., to the paper at a distance of 5 cm. from one end of the cylinder and at least 6 cm. away from the joined edges, since the solvent flow tends to be irregular near the join. After the test solutions had been allowed to evaporate on the paper at room temperature, the paper cylinder was stood in a glass tank (15 × 32 × 45 cm.) the foot of which contained the developing solvent (500 ml.) to a depth of about 1·5 cm. The tank was closed by a vaselined glass plate which carried an inlet tube for the introduction of SO_2. After development of the chromatogram for 20–24 hr., during which time the solvent had risen some 30–34 cm., the solvent was removed from the paper by drying in an oven at 40° for 3 hr., a current of N_2 being passed through the oven to facilitate removal of the solvent. The dried paper was then sprayed with 0·44 % (w/v) $K_3Fe(CN)_6$ in 0·2 M-phosphate buffer pH 7·8 (James, 1948). Adrenaline and certain related substances are thus rendered visible in the form of red or reddish-purple oxidation products. Quantities of adrenaline and noradrenaline down to 5 μg. may be detected on the paper in this way.

Water-saturated phenol was used in all but one of the chromatograms as developing solvent. The phenol was first distilled from zinc dust at atmospheric pressure, a procedure recommended by Williams & Kirby (1948). These chromatograms were carried out in an atmosphere of SO_2, the gas being passed into the tank for some 10–15 min. after the introduction of the paper. By this procedure, the solvent was rendered strongly acidic and also strongly reducing and oxidation of the catechol amines was prevented or diminished. The use of SO_2 in this way was incidental to the development of a method for the quantitative separation of adrenaline and noradrenaline. *n*-Butanol saturated with 0·5 N-HCl was used as developing solvent on one occasion. The alcohol had first been rendered aldehyde-free by refluxing with NaOH and distilling.

RESULTS

Paper chromatograms of the tumour extract

Paper chromatograms using water- and SO_2-saturated phenol as solvent showed the presence in the tumour extract of four substances yielding red or purplish-red derivatives with the ferricyanide reagent. A rough estimation from the size and colour intensity of the spots suggested that the four substances were present in about equal proportions. The

Table 1. R_F *values* of the four substances present in adrenal-medullary tumour extract using water- and* SO_2*-saturated phenol or* n*-butanol saturated with* 0·5 N-HCl *as solvents*

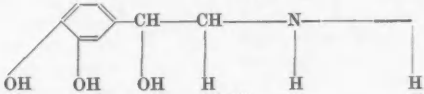
	R_F (phenol)	R_F (butanol)
Adrenal medullary tumour extract:		
Noradrenaline	0·27	0·14
Adrenaline	0·50	0·19
Compound *X*	0·62	0·55
Compound *Y*	0·75	0·58

* R_F value is the ratio distance of substance from starting point/distance of solvent front from starting point, and is here calculated on the distance from the starting point to the mid point of the spots.

position on the chromatogram of the compound with the slowest flow rate corresponded to that of noradrenaline while the position of the next line corresponded to that of adrenaline. Beyond the adrenaline position lay compound *X*, while still nearer to the solvent front lay compound *Y*. The spots of compound *X* and compound *Y* were V-shaped, indicating retardation of the flow of the main bulk of the material for some reason. Further reference will be made to this later. In Table 1, estimates of the R_F values of the four substances are given.

Comparison of the R_F *values of compounds* X *and* Y *with those of substances related to adrenaline and noradrenaline.* The possibility was first considered that compounds *X* and *Y* might be precursors of adrenaline or noradrenaline or some abnormal metabolites arising in the tumour issue. In an endeavour to identify them, the tumour extract was chromatographed in parallel with a number of possible candidates. The results for this chromatogram are given in Table 2. Reference to this table will show that a number of the substances had R_F values approximating to those of compounds *X* and *Y*. However, on repeating the chromatogram, but using *n*-butanol saturated with 0·5 N-HCl for irrigation of the paper, it was evident that compounds *X* and *Y* were not to be identified with any of the other substances.

Table 2. R_F *values of adrenaline and related compounds using water- and* SO_2*-saturated phenol or* n*-butanol saturated with* 0·5 N-HCl *as solvents*

Substance	Formula						R_F Phenol	R_F Butanol
								
Noradrenaline	OH	OH	OH	H	H	H	0·28	0·10
3:4-Dihydroxyphenylalanine	OH	OH	H	COOH	H	H	0·29	0·21
α-Ethylnoradrenaline	OH	OH	OH	C_2H_5	H	H	0·37	0·25
Hydroxytyramine	OH	OH	H	H	H	H	0·43	0·24
Corbasil	OH	OH	OH	CH_3	H	H	0·50	0·33
Adrenaline	OH	OH	OH	H	CH_3	H	0·51	0·17
Isoprenaline	OH	OH	OH	H	$CH.(CH_3)_2$	H	0·66	0·37
Epinine	OH	OH	H	H	CH_3	H	0·67	0·30
N-Ethylnoradrenaline	OH	OH	OH	H	C_2H_5	H	0·68	0·24
Adrenalone	OH	OH	=O	H	CH_3	H	0·69	0·24
N-Methyladrenaline	OH	OH	OH	H	CH_3	CH_3	0·72 (bleached spot no red coloration with the ferricyanide reagent)	0·16
Sympatol	OH	H	OH	H	CH_3	H	Not visible	Not visible
Metasympatol	H	OH	OH	H	CH_3	H	Not visible	Not visible

Evidence in favour of compounds X *and* Y *being derivatives of noradrenaline and adrenaline.* In the chromatogram run in *n*-butanol, adrenaline and noradrenaline in the tumour extract gave a combined spot paralleled in appearance and position by a mixture of adrenaline and noradrenaline chromatographed simultaneously. The lower half of the spot was coloured purplish-red and the upper half rose-pink as a result of the treatment with the ferricyanide reagent. The fact that compounds *X* and *Y* also gave a combined spot exactly similar in appearance to that of adrenaline and noradrenaline made it probable that compounds *X* and *Y* were similar derivatives of the two amines.

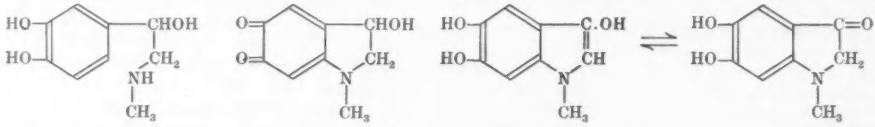
Evidence in favour of compounds X *and* Y *being esters of noradrenaline and adrenaline with the hydroxyl group of the side chains being involved.* The appearance of red-coloured pigments on treating adrenaline (I) or noradrenaline with the ferricyanide reagent is presumably the result of adrenochrome (II) and of noradrenochrome formation (see Bacq, 1949). The production of these compounds involves (*a*) oxidation of the catechol group to a quinone, and (*b*) formation of an indole nucleus by reaction of the benzene ring with the nitrogen atom of the side chain. Since compounds *X* and *Y* both yielded red pigments with the ferricyanide reagent it was therefore concluded, assuming the correctness of the hypothesis of their being adrenaline and noradrenaline derivatives, that the catechol group was still present and that the nitrogen atom of the side chain possessed a hydrogen atom since substitution in this latter position in the case of adrenaline would result in the production of a compound which could not form an indole derivative, as witness the fact that *N*-methyladrenaline did not give a red colour with the ferricyanide reagent (Table 2).

The evidence already discussed made it likely that compounds *X* and *Y* contained a free catechol group. Support for this belief was obtained from the following experiment.

A chromatogram of the tumour extract was developed in water- and SO_2-saturated phenol and dried thoroughly to remove all the phenol. It was then sprayed with an aqueous

solution of ferric chloride, a reagent which gives a green colour with catechols. The appearance of four green spots on the paper in the positions occupied by the four red spots obtained when the paper was sprayed with the ferricyanide reagent was indicative that not only adrenaline and noradrenaline, but also compounds *X* and *Y* contained a catechol group.

Thus it appeared probable that, of the functional groups of the adrenaline and noradrenaline molecules, the side chain hydroxyl group was the most likely point of substitution in the formation of compounds *X* and *Y*. Further presumptive evidence of this lay in the observation that, if the chromatogram of the tumour extract, after spraying with the ferricyanide reagent, was examined under ultraviolet light, a green fluorescence was apparent only in the adrenaline and noradrenaline positions, no such fluorescence being evident at the positions occupied by compounds *X* and *Y*. According to Lund (1949), the fluorescent substance obtainable from adrenaline results from an intramolecular rearrangement of adrenochrome (II) to give adrenolutine (III and IV) which may exist partly in the enol (III) and partly in the keto form (IV) (Fig. 1). Such tautomerism



Adrenaline (I) Adrenochrome (II) Adrenolutine (III) enol form Adrenolutine (IV) ketoform

Fig. 1.

would not be possible if the side chain hydroxyl group of adrenaline were substituted in any way, and thus it might be expected that no fluorescent compound would be obtained from an adrenaline derivative in which the side chain hydroxyl was, for example, esterified. If compounds *X* and *Y* were indeed esters of noradrenaline and adrenaline, acid hydrolysis should yield the parent amines. This was found to be so.

Acid hydrolysis of compounds X *and* Y

Compounds *X* and *Y* were separated from adrenaline and noradrenaline by paper chromatography. 0·2 ml. tumour extract was applied as a series of 5 μl. drops 5 cm. from the end of a cylinder of Whatman no. 1 filter paper. The chromatogram was developed in water-saturated phenol in an atmosphere of SO_2 for 24 hr. After drying off the solvent a narrow strip was cut from the paper in the direction of the solvent flow. Spraying this strip with the ferricyanide reagent located compounds *X* and *Y* and the corresponding portions were cut from the main bulk of the paper. The materials in the two paper strips thus obtained were eluted with 0·01 N-HCl using a method suggested by Dent (1947). One end of each paper strip was sandwiched between two microscope slides held together by an elastic band. The far sides of the slides were immersed in the elution fluid which passed by capillarity between the microscope slides and then on to and down the paper, the fluid dropping from the end of the paper strip being collected in a test tube. The set-up was covered by a glass tank to prevent undue evaporation of fluid from the paper surface. Elution was allowed to proceed overnight. The eluates were extracted with 3 equal vol. of benzene to remove traces of phenol and evaporated to dryness *in vacuo*. Each residue was dissolved in 0·5 ml. 0·01 N-HCl (unhydrolysed preparation). To effect hydrolysis 0·2 ml. of each solution thus obtained was placed together with 0·2 ml. 2 N-HCl in a small tube which was evacuated, sealed and heated in a boiling-water bath for 30 min. (hydrolysed preparation).

The hydrolysed and unhydrolysed preparations from compounds *X* and *Y*, together with the original tumour extract for comparison, were chromatographed on paper using water- and SO_2-saturated phenol as solvents. Spraying of the developed and dried chromatogram with the ferricyanide reagent gave the following results. From the hydrolysed preparation of *X* a red spot was obtained in a position corresponding with that of noradrenaline. No other spot was visible. The unhydrolysed preparation of *X* gave two spots, one in the noradrenaline position and the other at a position with an R_F value of 0·73. The hydrolysed preparation of *Y* gave only one spot, in the adrenaline position, while the unhydrolysed preparation yielded two spots, the first in the adrenaline position, and the second in a position with an R_F value of 0·90.

It was evident from this experiment that compound *X* was a derivative of noradrenaline and compound *Y* a derivative of adrenaline. Their behaviour to acid hydrolysis was consistent with the hypothesis of their being esters. The presence of the parent amines in the unhydrolysed preparations of compounds *X* and *Y* was probably to be explained by a partial hydrolysis occurring during the evaporation of the acid eluates from the paper strips. It will be noted that the R_F values of compounds *X* and *Y* in the unhydrolysed preparations were somewhat higher than those found when the tumour extract was chromatographed (see Table 1). It appeared likely that the preliminary chromatogram used to separate the compounds from the tumour extract had removed, partially or completely, material which retarded the flow of these substances. The outlines of the spots obtained from the unhydrolysed preparations of compounds *X* and *Y* were more or less oval and not V-shaped as they were when the

tumour extract was used. It was subsequently discovered that a similar increase in the R_F values of compounds *X* and *Y* could be effected in another way.

Separation of compounds X *and* Y *from adrenaline and noradrenaline by use of n-octyl alcohol.* In the course of the preparation of one batch of tumour extract, 0·5 ml. *n*-octyl alcohol was added to overcome troublesome frothing encountered during the evaporation of the acid alcohol solution *in vacuo*. The final extract contained two layers, an upper brown octyl alcohol layer and a lower yellow aqueous layer, the former being found to contain the compounds *X* and *Y* and the latter the adrenaline and noradrenaline. Compounds *X* and *Y* could be transferred to aqueous solution by dilution of the octyl alcohol with ether or benzene and extracting with 0·01 N-HCl. A chromatogram of such a solution gave R_F values for compounds *X* and *Y* of 0·79 and 0·89 respectively.

The probable structure of compounds X *and* Y

The question now arose as to what was the exact chemical composition of compounds *X* and *Y*. Unfortunately, adequate characterization of the pure substances was not possible since lack of material precluded their isolation. As a candidate for the position of the esterifying carboxylic acid, lactic acid came first to mind in view of Kendall's (1932) report of the presence of lactyladrenaline in extracts of adrenal glands. Kendall, however, stated that, 'no method was found to liberate adrenaline by hydrolysis'. This was not true of compound *Y*. The presence of a lactyl group in compounds *X* and *Y* might be demonstrated in two ways, (*a*) by preparing the lactyl derivatives of adrenaline and noradrenaline and showing that they behaved in a manner similar to that of compounds *X* and *Y* on a paper chromatogram and (*b*) by testing for lactic acid in the hydrolysed preparations of compounds *X* and *Y*.

Preparation of lactyladrenaline and lactylnoradrenaline. These were prepared according to a method outlined by Kendall (1932) for the preparation of lactyladrenaline. To about 5 mg. (−)-adrenaline or its equivalent of (±)-noradrenaline hydrochloride in a small dry glass tube were added 0·2 ml. methyllactate and a minute drop of conc. H_2SO_4 from a capillary pipette. The tubes were evacuated, sealed and heated in a boiling-water bath for 30 min. After cooling, the tubes were opened and the contents diluted with an equal volume of water.

Comparison of the R_F *values of lactyladrenaline and lactylnoradrenaline with those of compounds X and* Y. From each of the lactyl preparations two red spots were obtained on chromatography on paper with water- and sulphur dioxide-saturated phenol and subsequent spraying of the dried chromatogram with the ferricyanide reagent. In neither case was there any evidence of the presence of the parent amine. The R_F values of the two materials in the adrenaline preparation were 0·82 and 0·92 and of those in the noradrenaline 0·61 and 0·79. In explanation of the occurrence of two substances in these preparations it is tentatively suggested that one might have been an ester of lactic acid while the other was a derivative of lactyl-lactic acid. Whether this explanation be correct or not, the esterification of the amines had resulted in the formation of compounds which behaved on paper in a manner similar to compounds *X* and *Y* (R_F 0·79 and 0·89 respectively) obtained by the octyl alcohol method of extraction described above.

Demonstration of the presence of a lactic acid moiety in compounds X *and* Y. Lactic acid in solution may be detected by the reddish-violet colour which is obtained by the interaction of *p*-hydroxydiphenyl and conc. sulphuric acid with the acetaldehyde resulting from the treatment of lactic acid with hot concentrated sulphuric acid (Feigl, 1947).

To test for the presence of a lactyl radical in compounds *X* and *Y* the following experiment was performed. The compounds were separated from adrenaline and noradrenaline in 0·4 ml. tumour extract by paper chromatography as described above. The eluates containing *X* and *Y* were combined as were those containing the adrenaline and noradrenaline in order to have sufficient material to give an unequivocal result. The combined eluates were evaporated *in vacuo* to a volume of about 1 ml. Conc. HCl (0·1 ml.) was added to each and the solution heated under reflux for 30 min. to effect hydrolysis. The solutions were then further evaporated *in vacuo* to approximately 0·1 ml. and tested for lactic acid. Two drops of each test solution were heated with 1 ml. conc. H_2SO_4 in a dry test tube at 85° for 2 min. After the tubes had been cooled to about 30° under the tap, a small amount of solid *p*-hydroxydiphenyl was added to each solution which was then gently shaken and allowed to stand. A parallel test with pure lactic acid was also performed. On examining the tubes 30 min. later, positive reactions were observed in the tube containing lactic acid, and in the tube containing the hydrolysate from the preparation of compounds *X* and *Y*. The reaction in the 'adrenaline-noradrenaline' tube was negative.

These results were, therefore, indicative of the presence of a lactic acid moiety in compounds *X* and *Y*. This evidence is not conclusive, however, since pyruvic acid and α-hydroxybutyric acid also give a positive reaction (Feigl, 1947).

Compounds X *and* Y *as artifacts of the extraction technique*

Apart from Kendall's (1932) communication, there has apparently been no report in the literature of the occurrence of adrenaline or noradrenaline in the body in compound form of the type exemplified by lactyl-adrenaline. The possibility, therefore, of the formation of compounds *X* and *Y* in the course of the preparation of the extract of the tumour had to be investigated. Indicative that such was the case was the fact that, if the acid-alcohol extract of the tumour was evaporated to a small volume but not to dryness, the fluid contained only adrenaline and noradrenaline. Further evidence of artifact forma-

tion was provided by the observation that the solution obtained from a mixture of adrenaline and lactic acid put through precisely the same procedure as used in the preparation of the tumour extracts, contained, in addition to adrenaline, a substance which behaved on a paper chromatogram in a manner exactly similar to compound *Y* of the tumour extract. Thus, given the presence of adrenaline and lactic acid in the tumour, the indications are that the effect of the extraction procedure would be to produce some of compound *Y*. Noradrenaline might be expected to behave similarly.

Some observations on the pharmacological activities of compounds X *and* Y *and lactyladrenaline and of their hydrolysis products*

The pharmacological activity of these preparations were compared using the rat uterus test described by de Jalon, Bayo & de Jalon (1945) as modified by Gaddum, Peart & Vogt (1949) and by Gaddum & Lembeck (1949). Contraction of rat uterus, suspended in a small bath containing modified Ringer-Locke solution, is produced every 2 min. by the addition of a choline ester (acetylcholine or carbaminoylcholine). The addition of adrenaline or noradrenaline to the bath 1 min. before an addition of choline ester inhibits the contraction to a degree depending, within limits, on the amount of catechol amine added. The method can thus be used for the quantitative determination of the adrenaline or noradrenaline content of solutions.

An aqueous solution containing compounds *X* and *Y* free from adrenaline and noradrenaline was obtained from the tumour by the octyl alcohol method described above. Part of this solution was hydrolysed with N-hydrochloric acid by the method described. The activities of the hydrolysed and the unhydrolysed solutions were then compared on the rat uterus preparation. It was found that the unhydrolysed material had an activity of only about 2% of that of the hydrolysed material. A like figure was obtained with a preparation of authentic lactyladrenaline. Blocking of the side chain hydroxyl group by ester formation thus decreases markedly the pharmacological activity of adrenaline in this test. A similar reduction in activity is obtained if the side chain hydroxyl of adrenaline is oxidized to a ketone group (Gaddum *et al.* 1949).

SUMMARY

1. Evidence is presented of the presence of lactyladrenaline and lactylnoradrenaline in addition to adrenaline and noradrenaline in an extract of an adrenal medullary tumour.

2. The esters are believed to be artifacts produced by the method of extraction employed.

3. A pharmacological test demonstrated that these esters are relatively inactive compared with the parent amines.

I wish to express my thanks to Prof. G. W. Pickering for supplying the adrenal tumour, and to Prof. J. H. Gaddum, F.R.S., for his constant interest and encouragement throughout the course of this work.

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The Preparation and Properties of β-Glucuronidase

1. THE FRACTIONATION OF BUFFERED WATER HOMOGENATES

By LYNDA M. H. KERR and G. A. LEVVY
Rowett Research Institute, Bucksburn, Aberdeenshire

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For the assay of β-glucuronidase in animal tissues, it has been customary to prepare acid extracts from the water homogenates. Thus Talalay, Fishman & Huggins (1946) bring the homogenate to pH 5 with acetic acid, and incubate for 30 min. before removing insoluble material on the centrifuge. Kerr, Graham & Levvy (1948) follow a similar procedure, but adjust the pH to 5·2 with citrate buffer. Incubation at acid pH makes precipitated material more compact, but should obviously be kept as brief as possible if the final glucuronidase activity of the extract is to be taken as representative of that of the fresh tissue.

Becker & Friedenwald (1949) found that part of the glucuronidase activity of rat liver is precipitated at acid pH. This finding, which has recently been confirmed with mouse liver by Morrow, Greenspan & Carroll (1949), introduces possibilities of complications in the enzyme assay, which must be taken into account in comparing results obtained by different workers. The possibility had also to be considered that in our studies of the enzyme in varying states of tissue proliferation (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949, 1950) we were dealing with changes in only one fraction of the total activity, or with a transfer of activity from one fraction to another.

A study has been made of various factors entering into the preparation of tissue extracts which influence the results obtained in glucuronidase assay. The findings cast some light on the physical state of the enzyme in the cell.

EXPERIMENTAL AND RESULTS

General methods

Preparation of the enzyme. The tissue, usually mouse liver, was ground with water for 1 min. in a glass homogenizer. Water and buffer solution were added to the homogenate so that the final preparation was 0·1 N with respect to the buffer and contained 1 g. of moist tissue in 10 ml. ('10% preparation'). The pH of the preparation was checked with the glass electrode. Centrifuging was done on the M.S.E. Minor (Measuring and Scientific Equipment Ltd., London, S.W. 1) at about 1500 *g* for 15 min. Sedimented material was washed on the centrifuge with one-tenth of the original volume of 0·1 N-buffer, and this was added to the supernatant. For assay, the sediment was resuspended in 0·1 N-buffer with the aid of the glass homogenizer.

Enzyme assay. This was done by a modification of the method of Talalay *et al.* (1946), using phenolphthalein glucuronide as substrate. Throughout the assay, the same type of buffer was used as in the preparation of the enzyme. All enzyme preparations, totals or fractions were diluted with 0·1 N-buffer to a final volume of 40 ml./g. of tissue ('2·5% preparation'), with pH adjustment if necessary to 5·2. To 0·5 ml. of this preparation were added 3 ml. of 0·1 N-buffer, pH 5·2, and 0·5 ml. of 0·01 M-phenolphthalein glucuronide solution at the same pH. After incubation at 37° for 1 hr., there were added 4 ml. of a buffer solution, pH 10·7, containing glycine (0·4 M), NaCl (0·2 M) and Na_2CO_3 (0·25 M). Karunairatnam (private communication) has found that addition of Na_2CO_3 increases the efficiency of the glycine buffer. The mixture was centrifuged at 1500 *g* for 15 min., and the intensity of the red colour in the supernatant read with a Spekker photoelectric absorptiometer, using Ilford no. 605 yellow-green filter. Results are quoted as glucuronidase units (G.U.)/g. moist tissue, 1 G.U. liberating 1 μg. of phenolphthalein under the conditions described.

Fractionation of buffered homogenates

Table 1 shows the fractionation of the enzyme in an unincubated mouse-liver homogenate which was obtained when it was centrifuged after pH adjustment to 5·2 with acetate or citrate buffer. Sufficient animals were used in each experiment to keep the tissue concentration in the homogenate 10%. In many of the experiments, the preparation was kept ice-cold on the homogenizer and centrifuge, but omission of this precaution did not influence the fractionation. Homogenizing straight away in 0·1 N-buffer had no effect on the fractionation. Measurements of total activity in the unfractionated homogenate agreed with the sums of the two fractions.

It can be seen that in either acetate or citrate buffer just over 40% of the enzyme activity in normal adult liver was precipitated with the cell debris. The activity figure in citrate was always about 10% lower than the corresponding acetate figure, showing slight inhibition of glucuronidase by the former buffer (see Oshima, 1936; Mills & Paul, 1949). The glucuronidase activities of infant liver and of adult liver regenerating after partial hepatectomy have been shown to be high in comparison with normal adult values (Levvy *et al.* 1948). It would now appear that the differences in enzyme activity are confined to the soluble fraction, as shown

in Table 1. The percentage increase in activity observed on passing from a normal adult to an infant or regenerating tissue will thus be smaller if the assay figure includes the invariable activity of the debris.

Bringing the pH of a homogenate to 5·2 with hydrochloric acid, or by the addition of other buffers than acetate or citrate (phosphate, propionate, lactate, succinate or phthalate) led to a similar fractionation to that shown in Table 1.

When unbuffered homogenates (pH about 6·3) were centrifuged at 1500 *g*, the small precipitate obtained had no glucuronidase activity. Subsequent buffering of the supernatant to 5·2 with acetate then gave rise to the usual fractionation of the enzyme. As shown in the next paper in this series (Walker & Levvy, 1950), identical fractionation of the enzyme in such a supernatant can be obtained without prior pH adjustment on the high-speed centrifuge at 20,000 *g*. Microscopic study reveals the active precipitate to be granular in character. Addition of acetate to suspensions leads to clumping of the granules and easier sedimentation. In fact, slow sedimentation occurs in acetate under the action of gravity alone.

Effect of varying the pH *and concentration of the buffer*

Table 2 shows the effect of varying the pH of acetate or citrate buffer (0·1 N) on the fractionation of the enzyme in mouse liver. With acetate buffer, the partition of the enzyme between debris and supernatant did not change appreciably over the pH range 5·0–5·6. With citrate buffer, on the other hand, the fraction of the total enzyme activity which was precipitated decreased when the pH was raised beyond 5·2. In the higher pH range, the enzyme in the debris proved to be extractable on washing with

Table 1. *Fractionation of mouse-liver glucuronidase in buffered homogenates,* pH 5·2

(Substrate, phenolphthalein glucuronide. Values shown as mean ±S.E., followed in parentheses by the number of experiments. G.U., glucuronidase units: for definition see text.)

	Glucuronidase activity (G.U./g. tissue)			
	Acetate buffer		Citrate buffer	
Description	Supernatant	Debris*	Supernatant	Debris*
Normal adult	1550±130 (14)	1140±70 (14)	1390±120 (14)	1030±70 (14)
Infant (2–8 days old)	4190±210 (7)	1110±100 (7)	3960±340 (6)	1030±100 (6)
Adult, 4 days after partial hepatectomy	2880±100 (6)	1110±30 (6)	2670±40 (6)	1020±20 (6)

* Washed thrice on the centrifuge.

Table 2. *Effect of* pH *of the buffer on the fractionation of mouse-liver homogenate*

(Buffer concentration 0·1 N. Substrate, phenolphthalein glucuronide. Results shown as % of initial homogenate activity.)

	Acetate buffer			Citrate buffer		
pH	Initial supernatant	Initial debris*	Debris after washing†	Initial supernatant	Initial debris*	Debris after washing†
5·0	61	39	37	62	42	39
5·2	58	45	43	62	44	43
5·4	61	42	43	66	39	23
5·6	62	44	42	89	20	9

* Washed once on the centrifuge. † Additional three washings on centrifuge.

Table 3. *Effect of varying the concentration of the buffer* (pH 5·2) *on the fractionation of mouse-liver homogenate*

(Substrate, phenolphthalein glucuronide. Results shown as % of initial homogenate activity.)

	Acetate buffer			Citrate buffer		
Buffer concentration (N)	Initial supernatant	Initial debris	Debris after washing*	Initial supernatant	Initial debris	Debris after washing*
0·5	61	39	38	78	23	13
0·25	61	41	41	68	34	31
0·1	61	39	38	56	47	43
0·05	62	39	38	53	45	40
0·025	62	41	38	54	45	40

* Washed thrice on centrifuge.

citrate, but not with acetate. Further experiments showed that extraction of the granular enzyme with citrate buffer, pH 5·4–5·6, followed the same course whether the initial precipitation was done in acetate or citrate. Of other buffers mentioned in the preceding section, the one which most closely resembled citrate for extraction purposes was phosphate.

From Table 3 it can be seen that as the concentration of citrate buffer increased at constant pH (5·2) so did its efficiency in extracting the debris enzyme. Varying the acetate concentration had no effect of any kind on the fractionation.

Changes in the partition of the enzyme during incubation of homogenates

The water homogenate was buffered to pH 5·2 and incubated at 37°. At varying periods, samples were withdrawn for glucuronidase assay, with and without prior fractionation on the centrifuge.

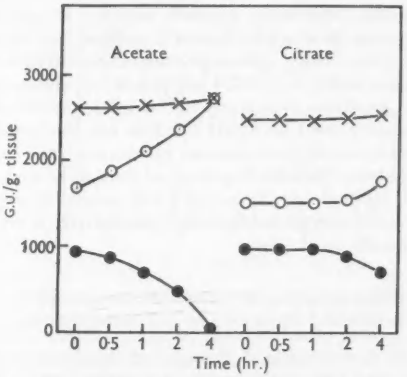

Fig. 1. Fractionation of homogenates of normal adult mouse liver after varying periods of incubation in acetate or citrate buffer, pH 5·2. Substrate, phenolphthalein glucuronide. ×—×, Total homogenate; ●—●, debris (washed thrice on centrifuge); ○—○, supernatant. (For definition of G.U. and details of assay procedure see text.)

Figs. 1–3 show results obtained with normal adult liver, infant liver, and liver regenerating after partial hepatectomy respectively. In acetate buffer, there was a progressive loss of enzyme activity from the granular material to the supernatant, with little change in the overall activity of the homogenate. This process was complete in about 4 hr. Figures for glucuronidase activity based on acetate extracts of the tissue thus depend entirely upon the duration of the incubation before removal of debris. Unless the time factor is strictly controlled, changes in the activity of the soluble enzyme *in vivo* may be obscured. With citrate buffer, any loss of enzyme from the debris to the supernatant was much less marked. A short period of incubation in this buffer can be seen to have little effect on the assay figure for the soluble enzyme fraction.

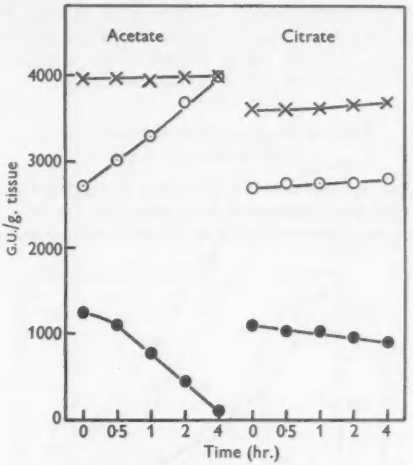

Fig. 2. Fractionation of homogenates of 5 day old mouse liver after varying periods of incubation in acetate or citrate buffer, pH 5·2. Substrate, phenolphthalein glucuronide. ×—×, Total homogenate; ●—●, debris (washed thrice on centrifuge); ○—○, supernatant.

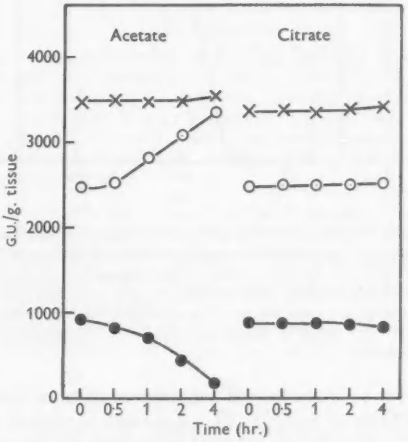

Fig. 3. Fractionation of homogenates of adult mouse liver 4 days after partial hepatectomy. Homogenate incubated for varying periods in acetate or citrate buffer, pH 5·2. Substrate, phenolphthalein glucuronide. ×—×, Total homogenate; ●—●, debris (washed thrice on centrifuge); ○—○, supernatant.

Supernatants separated prior to incubation showed no change in activity after incubation for

4 hr. in acetate buffer, pH 5·2. Longer periods of incubation sometimes resulted in partial inactivation. Fractionation of suspensions of washed granular material incubated at 37° revealed a fall in debris activity exactly as illustrated in Figs. 1–3, with the formation of equivalent amounts of soluble enzyme. Citrate again inhibited the transfer of activity from the debris to the supernatant.

Effect of varying pH *on the liberation of enzyme from the debris*

A study was made of the effect of varying the pH on the rate of liberation of soluble enzyme from the granules in acetate buffer at 37°. Washed mouse-liver

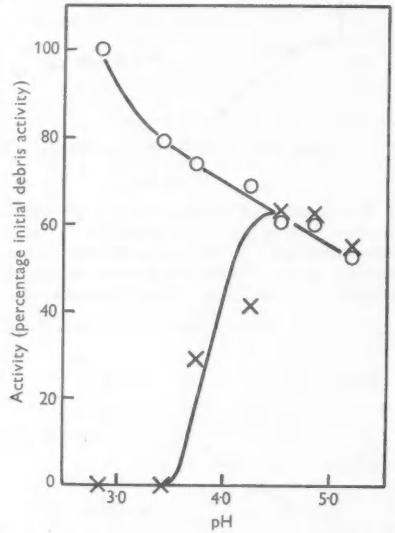

Fig. 4. Effect of varying pH on the rate of liberation of enzyme from washed mouse-liver debris in acetate buffer. Incubation for 1 hr., followed by separation and assay (phenolphthalein glucuronide) at pH 5·2. Results expressed as percentages of unincubated debris activity. O—O, Loss in debris activity; ×—×, supernatant activity.

debris, separated at pH 5·2, was suspended in buffer solutions of various pH values and incubated for 1 hr. After bringing the pH back to 5·2, the preparation was centrifuged and measurements made of the glucuronidase activity of the two fractions in acetate buffer. Fig. 4 shows the loss in debris activity and the gain in supernatant activity, both expressed as percentages of the initial debris value. As the pH fell from 5·2 to 4·5, there was a slight increase in the rate of transfer of enzyme from debris to supernatant. Below pH 4·5, enzyme inactivation became marked, and the loss in debris activity was no longer balanced by a gain in the supernatant. At pH 3·4, incubation for 1 hr. caused a fall of about 80 % in the debris activity, and completely inactivated any enzyme which had gone into solution.

Preparations of the soluble enzyme from unincubated homogenates were incubated for 1 hr. at various pH values in acetate buffer, with final assay after pH adjustment to 5·2. There was no fall in activity after incubation at pH 4·5–5·2. Below this, inactivation was observed, reaching 100 % at pH 3·4.

These experiments suggest that all the soluble mouse-liver glucuronidase is inactive in an assay following a preliminary incubation for 1 hr. in acetate buffer at pH 3·4. Mills & Paul (1949) and Mills, Paul & Smith (1949) have, however, reported the presence in tissue extracts of a glucuronidase with a pH optimum at 3·4. This enzyme was at first claimed to be specific for the hydrolysis of phenol glucuronide, but has since been found (Mills, private communication) to hydrolyse phenolphthalein glucuronide, also with optimal activity at pH 3·4. Experiments along the lines of those described above, with final assay (phenolphthalein glucuronide in acetate buffer) at pH 3·4 instead of 5·2, showed no activity whatsoever in any fraction after preliminary incubation for 1 hr. at pH 3·4. This was also true for unfractionated homogenates put through the same procedure. These findings suggest that, if the enzyme with optimal activity at pH 3·4 is present in mouse liver, it is very unstable at the optimum pH, in crude preparations at least.

Effect of varying the tissue concentration on the liberation of debris enzyme and in the final assay

As shown in Fig. 5, the rate of liberation of glucuronidase from the granular material during the incubation of a mouse-liver homogenate at 37° in acetate buffer, pH 5·2, was unaffected by the tissue concentration. Parallel experiments were done with the preparation diluted to 10 and to 40 %. At the last stage before incubation with substrate all preparations were brought to 2·5 %.

In the final incubation with substrate the activity figures for preparations containing insoluble enzyme were markedly influenced by variations in the concentration of tissue constituents. This is illustrated in Table 4. When 0·5 ml. of an unincubated homogenate in which the tissue concentration was 40 % was taken straight for assay in acetate, the final activity figure was only about half that obtained when the preparation was first diluted to 2·5 %. Low values for the activity were still obtained after fractionation of the 40 % preparation, particularly in the case of the insoluble fraction. Since, however, each fraction was brought to the same volume as that of the homogenate, there was a dilution of the tissue

at this stage, resulting in an apparent increase in the overall activity of the 40% preparation after fractionation.

When the debris enzyme was completely converted to the soluble form by preliminary incubation for 4 hr., variations in the tissue concentration no longer affected the rate of hydrolysis of the substrate. The 40% preparation thus appeared to show an increase in total activity during the incubation.

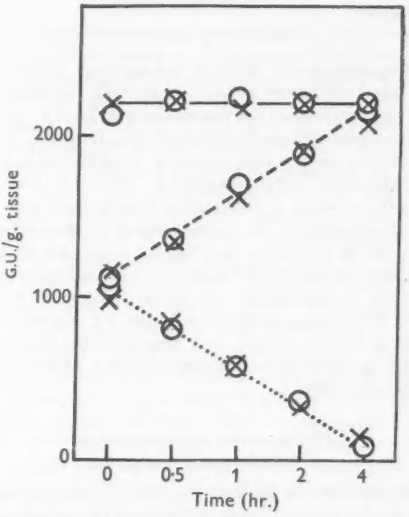

Fig. 5. Effect of varying the tissue concentration on the rate of liberation of enzyme from mouse-liver debris in acetate buffer, pH 5·2. Substrate, phenolphthalein glucuronide. ×, 40% homogenate; ○, 10% homogenate. —, Total; ---, supernatant; . . . . ., debris.

The tissue concentration factor makes the glucuronidase assay procedure of Kerr *et al.* (1948) inapplicable for present purposes, since the final enzyme concentration required in the incubation with phenol glucuronide corresponds to that resulting from the use of a 40% preparation in the assay with phenolphthalein glucuronide. On this account, a great many experiments carried out in the early stages of this work have had to be discarded. The apparent changes in the activities of unfractionated mouse-liver homogenates during incubation in acetate buffer, as measured with phenol glucuronide, are shown in Fig. 6. The homogenates were 10% at this stage. The addition of buffered substrate (Kerr *et al.* 1948) resulted in a twofold dilution (cf. the eightfold dilution in the assay with phenolphthalein glucuronide). Fig. 6 shows the effects of heat inactivation very clearly.

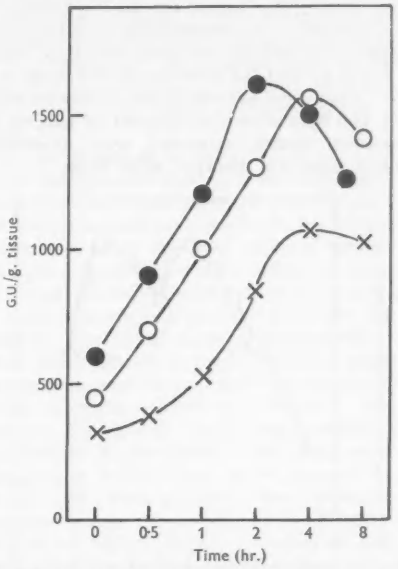

Fig. 6. Apparent change in the total activities of 10% mouse-liver homogenates during incubation at 37° in acetate buffer, pH 5·2. Substrate, phenol glucuronide in acetate buffer. ×—×, Normal adult; ●—●, 4 day old infants; ○—○, adult, 4 days after partial hepatectomy. See Kerr *et al.* (1948) for definition of G.U. and details of assay procedure.

It should perhaps be pointed out that our studies of glucuronidase in tissues in varying states of

Table 4. *Effect of varying the tissue concentration during the incubation with substrate*

(Acetate buffer, pH 5·2. Substrate, phenolphthalein glucuronide. Normal mouse liver. G.U., glucuronidase units; for definition see text.)

Final concentration of tissue (g. moist tissue/100 ml.)	Glucuronidase activity (G.U./g. tissue)					
	No preliminary incubation			Preliminary incubation for 4 hr.		
	Total	Supernatant	Debris*	Total	Supernatant	Debris*
40	1100	815	628	1920	1930	0
8	2080	1100	990	2110	2060	0
2·5	2040	1080	1050	2020	1940	0

* Washed thrice on centrifuge.

proliferation, referred to in the Introduction, were carried out with preparations of the soluble enzyme fraction, after partial purification by precipitation with ammonium sulphate.

pH-activity curve for the insoluble enzyme

The pH-activity curve for the hydrolysis of phenol glucuronide by preparations of soluble mouse-liver glucuronidase has two peaks in citrate buffer, one at pH 4·5 and the other at pH 5·2 (Kerr *et al.* 1948). Separation into two fractions corresponding to the two optima can be achieved by making the preparation 38·5% saturated with ammonium sulphate (Kerr *et al.* 1949; cf. Mills, 1948).

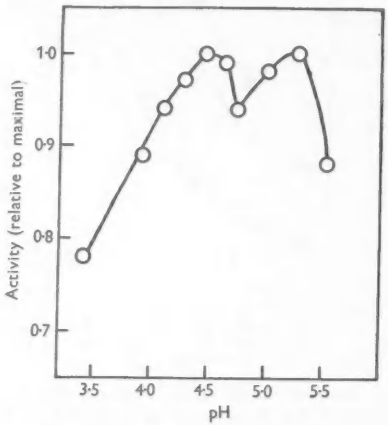

Fig. 7. pH-Activity curve for the insoluble glucuronidase fraction in mouse liver. Unbuffered homogenate freed from inactive material by centrifuging; active material precipitated with acetate buffer, pH 5·2, washed on centrifuge and disintegrated by shaking with Ballotini.

As shown in the next paper in this series (Walker & Levvy, 1950), the granular material obtained from water homogenates of mouse liver can be disintegrated by shaking at a high speed with Ballotini, Grade 12 (Chance Bros Ltd), without inactivation of glucuronidase. By this method, the enzyme in the granules can be brought into solution, presumably without chemical change. As can be seen from Fig. 7, the resulting preparation gave a pH-activity curve for the hydrolysis of phenolphthalein glucuronide in acetate buffer with peaks at pH 4·5 and 5·2. Preparations of the soluble enzyme from unincubated homogenates gave similar curves. It is evident that the fractionation of the enzyme brought about by centrifuging at pH 5·2 does not coincide with that obtained after partial precipitation with ammonium sulphate.

The action of saccharate

Suspensions of washed mouse-liver debris in acetate buffer were almost completely inhibited by 10^{-2} M-saccharate (Karunairatnam & Levvy, 1949). This concentration of saccharate was, however, without effect on the conversion to soluble enzyme in acetate buffer at 37° and pH 5·2. After 2 hr. incubation in presence of saccharate, the residual activity (42% of initial) of the washed debris was the same as in controls incubated in absence of saccharate. The soluble enzyme formed was freed from saccharate by twofold precipitation with equal volumes of saturated ammonium sulphate solution (cf. Karunairatnam & Levvy, 1949). The final activity was once again the same as in the controls (59% of the initial debris activity).

Fractionation of the enzyme in kidney and uterus

Table 5 shows the fractionation of the enzyme in mouse kidney and uterus obtained by centrifuging the unincubated water homogenates in acetate buffer, pH 5·2, at 1500*g*. The tissue concentration at this stage was 10% with kidney and 2% with uterus. After suitable dilution of the two fractions, the assay was carried out in acetate buffer at pH 5·2 for kidney and 4·5 for uterus (cf. Kerr *et al.* 1949). In kidney, as in liver, the higher glucuronidase activity of the infant tissue as compared with adult (Levvy *et al.* 1948) is seen to be confined to that fraction of the enzyme which was initially soluble. As regards the debris enzyme, the difference between the average figures for infant and adult kidney was not statistic-

Table 5. *Fractionation of the enzyme in mouse kidney and uterus in* 0·1 N-*acetate buffer*, pH 5·2

(Substrate, phenolphthalein glucuronide. Values shown as mean ±S.E., followed in parentheses by the number of experiments. G.U., glucuronidase units; for definition see text.)

Description of tissue	Glucuronidase activity (G.U./g. tissue) Supernatant	Debris*
Kidney,† normal adult	1510±90 (6)	1350±90 (6)
Kdney,† 5 days old	2850±270 (3)	1090±140 (3)
Uterus,‡ ovariectomized adult	920±40 (5)	370±30 (5)
Uterus,‡ 4 days after subcutaneous injection of 1·7 mg. oestrone/kg. in ovariectomized adult	2130±120 (6)	390±30 (6)

* Washed thrice on centrifuge. † Assay at pH 5·2. ‡ Assay at pH 4·5.

ally significant ($P > 0{\cdot}05$). In the adult organ, this fraction of the enzyme accounted for nearly 50% of the total activity. Uterus also contained an insoluble glucuronidase fraction which did not vary with the state of proliferation of the tissue (Table 5). The rise in uterine glucuronidase activity which follows the injection of ovariectomized mice with oestrone (Kerr *et al.* 1949) was confined to the supernatant.

DISCUSSION

No evidence from the present investigation causes us to modify our view that the β-glucuronidase activity of a tissue reflects its state of proliferation (Levvy *et al.* 1948; Kerr *et al.* 1949, 1950). It now appears, however, that only a fraction of the enzyme, accounting with normal mouse liver and kidney for slightly more than half the total activity, varies in this respect. The existence of the other insoluble fraction introduces complications into the assay that make it difficult to compare results obtained by different methods. The procedure used by Talalay *et al.* (1946) in preparing the enzyme for assay leads to the discard of an amount which varies with the period of incubation of the homogenate in the acetate buffer. This may explain some of the difficulties encountered by Mills *et al.* (1949) in attempting to repeat some of our earlier work, since they incubated the homogenate in acetate buffer, pH 5, for 2 hr. or more, depending upon the volume, and then discarded insoluble material (Mills, private communication). It is possible that the greater part of the enzyme activity initially present in the granules was obtained in the final extract, but inclusion of this fraction in the activity figures makes differences between normal adult and actively proliferating tissues smaller. Moreover, the autolytic process for release of debris enzyme is limited by heat inactivation and may be affected by other, unknown variables. It should, however, be made clear that the experiments in question were confined to rats (Mills, private communication). The most suitable procedure for following changes in the glucuronidase activity of a tissue *in vivo* would appear to be the use of the supernatant obtained by centrifuging an unincubated homogenate in acetate buffer, pH 5·2.

Provided that the pH and concentration are strictly controlled the use of citrate buffer as in the procedure of Kerr *et al.* (1948) gives activity figures representing the soluble glucuronidase fraction, even after the homogenate has been incubated for short periods. This buffer has, however, the disadvantage that slight alterations in pH lead to extraction of the debris enzyme. It should be pointed out that the detailed assay procedure of Kerr *et al.* (1948), in which concentrated citrate buffer, pH 5·2, is added to the homogenate, frequently results in a final pH of 5·3–5·4. Fortunately, it has been our practice in studying changes in glucuronidase activity to check the pH of the buffered homogenate, and if necessary adjust it to 5·2 before incubating and centrifuging. Another feature of the assay procedure of Kerr *et al.* (1948) is the assumption that the activity in a centrifuged homogenate is evenly distributed between precipitate and supernatant before decanting, so that a sample of the latter may be taken as representative of the whole. We have found that this is only true in so far as it refers to that fraction of the total enzyme which is soluble at the pH of the citrate buffer.

In the Discussion to an earlier paper (Kerr *et al.* 1950), mention was made of preliminary findings in the present investigation, and it was suggested that the active insoluble material contains a precursor of the enzyme. This view was based on experiments with phenol glucuronide as substrate, which showed an increase in the total activity of the homogenate as more enzyme was released from the granular material. For reasons already discussed, the phenol glucuronide method is unreliable when applied to preparations containing insoluble enzyme. More recent experiments leave us no reason to suppose that its insolubility makes this glucuronidase fraction any the less active in the living tissue. Indeed, if the granules are, as we suspect, mainly mitochondria, their glucuronidase activity can be demonstrated in the intact cell by histochemical methods (Campbell, 1949). Bernfeld & Fishman (1950) have observed activation of glucuronidase by deoxyribonucleate, and suggest that this largely determines the activity of the enzyme in a tissue. On this view, there is no need to imagine the existence of preformed enzyme in an inactive state to explain the increase in the glucuronidase activity of a tissue during active proliferation.

SUMMARY

1. Addition of acetate buffer, pH 5·2, to water homogenates of mouse liver, kidney and uterus causes agglutination of granular material which can then be separated by centrifuging at low speeds. This material has glucuronidase activity, amounting with normal adult liver and kidney to slightly less than half the total in the homogenate.

2. Citrate buffer causes identical fractionation of the enzyme in homogenates, provided that the pH and concentration are strictly controlled. Increasing the pH beyond 5·2 or the concentration beyond 0·1 N leads to extraction of the enzyme in the granules by citrate.

3. Changes in the glucuronidase activity of a tissue *in vivo* are confined to that fraction of the enzyme which is soluble in acidified water homogenates.

4. Incubation of homogenates in acetate buffer leads to a progressive release of enzyme from the granules to the solution. This does not occur in citrate buffer.

5. In concentrated homogenates, the presence of the insoluble enzyme fraction can lead to false results for the total activity.

6. The pH-activity curves for the hydrolysis of phenolphthalein glucuronide in acetate buffer by the soluble and insoluble fractions are identical in having peaks at pH 4·5 and 5·2.

7. Saccharate inhibits the enzyme in the granules without affecting its release to the solution on incubation in acetate buffer.

8. The significance of these findings in the assay of glucuronidase is discussed, with particular reference to tissues in varying states of proliferation.

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The Micro-estimation of Benzoic and Hippuric Acids in Biological Material

By F. DICKENS and JOAN PEARSON

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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In a study of the aromatization of *cyclo*hexanecarboxylic acid and related compounds by animal tissues (Beer, Dickens & Pearson, 1950) it was necessary to be able to estimate small amounts of benzoic or hippuric acids in presence of other organic acids, and particularly of the unaromatized substrate employed. For this purpose existing methods were not applicable, and in this paper a method is described which has been found adequate for the conditions of the above experiments. The method is based on the nitration of benzoic acid at room temperature, reduction of the resulting mononitrobenzoic acid to aminobenzoic acid, diazotization of the latter and coupling with a colour-forming base. The estimation is completed by colorimetric estimation of the resulting dye and is here described for application to samples containing 0·05–10 mg. benzoic acid or equivalent amounts of hippuric acid.

Older methods of estimation of benzoic and hippuric acids

Urinary hippuric acid has been estimated by isolation and weighing (Friedmann, 1911); a method only suitable for large quantities. The titrimetric procedures of Folin & Flanders (1912) and Kingsbury & Swanson (1921) required continuous extraction of the urine with ether and titration of the benzoic acid liberated on hydrolysis of the hippuric acid. Quick (1926) determined instead the amino nitrogen by formol titration. Griffith (1926; see also Hawk & Bergeim, 1938) destroyed traces of urea in the ether extract and then estimated hippuric acid nitrogen by Kjeldahl analysis. None of these methods is suitable for the present purpose, and the latter two methods cannot be used for estimation of benzoic acid in any case.

Colorimetric methods depending on aromatic structure would obviously be preferable. Nicholls (1928) oxidized benzoic acid by hydrogen peroxide to salicylic acid which can be colorimetrically estimated; this oxidation proceeds only to the extent of about 10%, and is therefore an unsatisfactory basis for a quantitative method.

The most promising published micromethod for benzoic acid estimation appeared to be that of Waelsch & Klepetar (1935) and Waelsch & Busztin (1937). This is based on the hot nitration of benzoic acid to give a dinitrobenzoic acid, thought (Mohler,

1890) to be 3:5-dinitrobenzoic acid. Grossfield (1915) reduced the nitration product in alkaline solution with hydroxylamine to give a red colour, and the reaction was developed by the Prague workers into a quantitative test for amounts of 0·1–50 mg. benzoic acid. This reaction was also found to be unsuitable for the present purpose since *cyclo*hexane compounds (and many other substances also) char to such an extent in the hot nitrating mixture that no reading of the final colour is possible. Moreover, the method involves the selection of a time of maximum colour development (10–55 min.) at which readings must be taken, which is dependent on the actual amount of benzoic acid present. Further drawbacks were found to be the non-linearity of colour development with concentration of benzoic acid and the fact that we have found (Beer, Dickens & Salmony, 1950) that the true colour former in this reaction appears to be mainly traces of 2:5-dinitrobenzoic acid and not the 3:5-dinitrobenzoic acid formed as the main product in the hot nitration of benzoic acid.

EXPERIMENTAL

Method for micro-estimation of benzoic and hippuric acids

Reagents. Nitrating mixture: a solution of 10 g. A.R. KNO_3 in 100 ml. H_2SO_4, 98% (w/w). $TiCl_3$ (13–15%, w/v) in protected burette. $NaNO_2$ A.R. 1 g./10 ml. water; freshly prepared solution.

Coupling reagent: *N*-(1-naphthyl)-ethylenediamine hydrochloride, 0·5 g./100 ml. N-HCl, solution used within 2 days of dissolving. *iso*Amyl alcohol, 'purified' or 'milk-testing' grade. NaOH (40 g./100 ml. water; approx. 9·6 N) and dilute (approx. 0·1 N) solution. Sodium carbonate 20% (w/v) Na_2CO_3; solid urea and tartaric acid; HCl, 32% (w/w).

Procedure. (1) The sample, which should not exceed 10 ml. in volume or contain more than 10 mg. benzoic (or hippuric) acid, is measured into a 20 ml. glass or porcelain crucible, made alkaline if necessary with a few drops excess of 20% sodium carbonate and evaporated to dryness on a steam bath. The drying is completed in the oven for 30 min. at a temperature not exceeding 100°.

(2) The cooled, perfectly dry residue is nitrated at room temperature for 30 min. by addition of 1·0 ml. nitrating mixture, with stirring to ensure solution. After this time the contents of the crucible are cautiously diluted with 3·5 ml. water and cooled. Partial neutralization is then effected by the addition of 2·5 ml. of the concentrated sodium hydroxide solution (see *Reagents*).

(3) The contents of the crucible are transferred with 3 ml. water to a 50 ml. separating funnel, or to a test tube, 20 × 130 mm., provided with a glass or rubber stopper and in addition with a suitable double-bored rubber stopper carrying a pair of fine glass tubes for blowing out the top layer (as in a wash-bottle): the depth of immersion of the longer glass tube is adjustable and its lower end is turned upwards. The use of these tubes is more convenient if series of analyses are done simultaneously, as they are readily rotated for mixing the contents by attaching them to a rotating disk provided with a double row of spring clips. Each tube has its own blow-out head as described above.

*iso*Amyl alcohol (10 ml.) is added to each tube or separating funnel, and, after thorough mixing, the upper layer of amyl alcohol is transferred to a 50 ml. separating funnel. A second extraction with 10 ml. amyl alcohol is added to the first. By using tubes of appropriate size emulsions which may form with some biological extracts are quickly separated by centrifugation: the above size is convenient for the small bench-type angle centrifuge used by us.

(4) The combined amyl alcohol extracts are washed by thorough shaking in the separating funnel with three successive portions each of 5 ml. water, the aqueous washings being rejected. The nitration product is then extracted from the amyl alcohol by thoroughly shaking with two portions, each of 5 ml. approx. 0·1 N-sodium hydroxide. These extracts, which must be alkaline in reaction, are transferred to a 50 ml. pyrex boiling tube.

(5) To the contents of the tube are added 15 ml. concentrated hydrochloric acid, 12 g. tartaric acid crystals and, after solution, 1 ml. titanous chloride solution. The whole is placed in a boiling-water bath for exactly 15 min., when the tube and contents are cooled to below 5° in ice. Diazotization follows, by gradual addition of 1 ml. 10% sodium nitrate and letting stand 30 min. in the ice bath.

(6) While the tube is still cooled in ice, urea (7 g.) is cautiously added to remove excess nitrous acid. It is best to pour the total quantity of solid urea into the tube without stirring, allow it to settle, and then stir to solution. This will avoid loss by frothing. After standing 10 min. the solution should be free of nitrous acid.

(7) The coupling reagent (5 ml.) is then added, the mixture removed from the ice bath and let stand 30 min. before being made up to exactly 100 ml. with water and 10 ml. concentrated hydrochloric acid. After a further 60 min. for colour stabilization, the density of the red colour is estimated in an absorptiometer provided with a green light source (Ilford spectrum green light filter no. 604, transmission 5000–5400 A. used with 1 cm. depth cell in the Hilger absorptiometer). If the amount in the original sample does not exceed 10 mg., solutions of too intense colour for direct measurement may, if necessary, be quantitatively diluted with water before reading, the readings obtained being multiplied by the appropriate dilution factor.

(8) Reagent blank. This is determined for each batch of reagents by blank estimations put through

the full procedure. The blank density reading with various batches (1 cm. cell, green filter) was 0·07–0·10 corresponding to a total amount of 'benzoic acid' of 0·035–0·050 mg. in our analyses. The use of pure ethanol (see below) for deproteinization did not affect this blank.

(9) Calculation. A calibration curve is prepared by estimations on known amounts of benzoic and hippuric acid over the range 0·05–10 mg. (see Fig. 1).

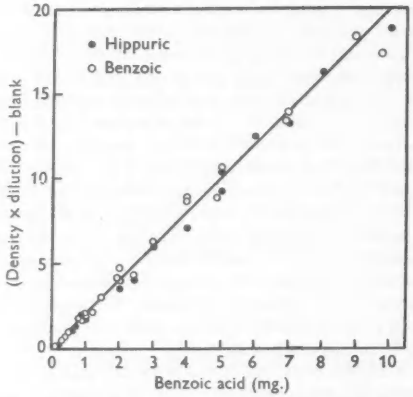

Fig. 1. The estimation of aqueous solutions of pure benzoic acid (○) and hippuric acid (●). Abscissae: amount in mg. of benzoic acid or of benzoic acid equivalent of the hippuric acid taken. Ordinates: absorptiometer density readings multiplied by dilution factor, corrected for reagent blank (see text).

This is sufficiently close to a straight line for a constant factor to be employed for calculation, the mean value of our calibration factor being 0·50. If the observed optical density (1 cm. cell, green filter) is d, the dilution factor (if any) is F and the density in the reagent blank is b, then:

$$\text{mg. benzoic acid in sample} = 0{\cdot}50\ (dF - b),$$

where 0·50 is the empirical value of the calibration factor determined as described above. Fig. 1 shows that hippuric acid is estimated by this method as the equivalent amount of benzoic acid; a point of practical convenience in the present method.

If it should be necessary to distinguish between benzoic and hippuric acids (it was not necessary in the work for which this method was devised), this could be done by separate estimations on the extracted acids of total benzoic acid (free and conjugated) by the above method, and also of glycine nitrogen as for example in the method of Griffith (1926).

Accuracy. The blanks on reagents were well duplicated, twenty-eight estimations on one set of reagents giving a mean value of $d = 0{\cdot}104 \pm$ S.E. 0·013. This would correspond to $0{\cdot}052 \pm 0{\cdot}007$ mg. benzoic acid, and is always subtracted from the readings as explained above.

There was more variation in the estimation of pure solutions of benzoic and hippuric acids, as would be expected in view of the fairly large number of operations concerned. Nevertheless, the method has proved more reliable than previous methods in our experience. The mean percentage errors were: over the range 0·1–1·0 mg. benzoic acid, −6 % (19 observations); hippuric acid, +3 % (10 observations): over the range 1–10 mg., benzoic acid, +4 % (23 observations); hippuric acid, −0·1 % (12 observations). Individual single estimations are scattered as shown in Fig. 1, but the above calculations show that there would be no practical advantage in using different factors for the calculation in different regions of the 0·1–10 mg. range or for the two different acids (all results are expressed as the equivalent amount of benzoic acid whichever acid is actually being estimated).

Application to tissue slices and homogenates

For the estimation of benzoic acid present in tissue preparations after incubation in manometric vessels, the proteins are first removed by precipitation with 60 % ethanol. The whole contents of the vessel (after removal of any carbon dioxide absorbent used) are rinsed out or diluted in the vessel with a measured volume of water to make 6·0 ml. total volume, which is made to 15 ml. with a sufficient quantity of pure absolute ethanol to give a final concentration of 60 % (v/v). After standing for at least 2 hr., and filtering through paper, a measured portion (usually 10 ml.) of the filtrate is transferred to a 20 ml. crucible. If interfering substances such as partially hydrogenated derivatives of benzoic acid are present they must be eliminated by bromination, otherwise the 10 ml. ethanolic filtrate is made just alkaline by addition of a few drops of 20 % sodium carbonate and evaporated to dryness. From this stage the analysis is as described above.

For removal of interfering substances, the 10 ml. ethanolic filtrate is adjusted, if necessary, to pH 6·8, using bromothymol blue as external indicator. Saturated bromine water (2 ml.) is then added, and after 30 min. at room temperature the excess bromine is blown off by a fine but rapid stream of nitrogen: the time for this should not exceed 15 min. The solution is then made just alkaline and evaporated as described in the preceding paragraph.

The recovery by this method of added benzoic and hippuric acids from samples of tissue slices is shown in Table 1.

Application to blood. To the samples of blood (5 ml.) 2 ml. water and sufficient absolute ethanol (10·5 ml.) are added to make the final volume after mixing 17 ml. After standing for 2 hr., measured

Table 1. *Recovery of added benzoic and hippuric acids from rabbit-tissue slices*

(Pooled tissue slices of moist wt. 300 mg. suspended in phosphate-Ringer solution. Benzoic (0·61 mg.) or hippuric acid (equiv. to 0·61 mg. benzoic acid) added as shown.)

	Liver		Kidney	
Acid added	Benzoic acid found, total (mg.)	Recovery (%)	Benzoic acid found, total (mg.)	Recovery (%)
None	0·028	—	0·027	—
None	0·033	—	0·040	—
None	0·040	—	0·032	—
Benzoic	0·630	97·7	0·725	113·4
Benzoic	0·557	85·7	0·635	98·7
Benzoic	0·645	100·0	—	—
Benzoic	0·601	93·0	—	—
Hippuric	0·609	94·3	0·615	95·5
Hippuric	0·616	95·4	0·675	105·3
Hippuric	0·616	95·4	0·660	102·8

portions (5–10 ml.) of the filtrate are taken for analysis, for which purpose they are made slightly alkaline and evaporated as already described.

Table 2. *Recovery of added benzoic and hippuric acids from rabbit blood*

(To 5 ml. samples of blood the stated amounts of sodium salts of benzoic or hippuric acid (expressed as the equiv. amount of benzoic acid) were added, and the analysis completed as in the text.)

	Addition		
Acid	Amount of benzoic acid equiv. (mg.)	Benzoic acid found, total (mg.)	Recovery (%)
None	—	0·125	—
None	—	0·119	—
Benzoic	1	0·993	87
Benzoic	2	2·084	98
Hippuric	1	0·976	86
Hippuric	2	1·880	88

Table 2 illustrates the recovery of added benzoic and hippuric acids from whole blood: there is a slight loss, possibly due to adsorption on the voluminous precipitate. It is possible that part of the values obtained on the original blood may be due to substances other than benzoic or hippuric acid, e.g. *p*-aminobenzoic acid, the amount (about 2 mg./100 ml. blood) being too small for identification by isolation.

Application to urine. Owing to the presence of phenols and other interfering substances in urine, preliminary treatment with bromine is necessary. The urine (5 ml.) is adjusted to pH 6·8 with bromothymol blue as external indicator. Saturated bromine water (5 ml.) is added and after standing for 30 min. the mixture is filtered. The filtrate, which must smell strongly of bromine, is freed from excess of the latter by passing a brisk stream of nitrogen bubbles through it for 15 min. The whole, or a measured portion if abnormally high amounts of benzoic or hippuric acids are known to be present, is transferred to a separating funnel (or, better, the stoppered extraction tubes described above) with 1 ml. N-hydrochloric acid and water to make a total volume of 10 ml. It is then extracted by shaking with three successive portions each of 10 ml. *iso*amyl alcohol. The combined extracts are washed once in a separating funnel with 5 ml. water, and then the benzoic and hippuric acids are shaken out with two portions each 5 ml. approx. 0·1 N-sodium hydroxide. The combined alkaline extracts are evaporated to

Table 3. *Recovery of added benzoic and hippuric acids from rabbit urine*

(With bromination (see text). 5 ml. portions of urine were taken with the addition as shown below. Suitable measured portions were taken for analysis, the results being expressed as total benzoic acid in 5 ml. urine.)

	Benzoic acid		Hippuric acid		
Added (mg.)	Benzoic acid found, total (mg.)	Recovery (%)	Equiv. benzoic acid added (mg.)	Benzoic acid found, total (mg.)	Recovery (%)
0	1·57	—	0	1·6	—
1·22	2·72	94	17·04	18·5	99
2·44	4·01	100	27·2	28·9	100
3·66	4·76	87	34·1	32·1	90
			34·1	36·0	101
			34·1	37·0	104

dryness in a 20 ml. crucible and the analysis proceeds from this stage as described above.

The recovery of added benzoic and hippuric acids from urine is illustrated by Table 3. It should be noted that in some species part of the benzoic acid excreted in the urine may exist as a benzoyl glucuronide (see Williams, 1947). In such cases it will be necessary to hydrolyse the benzoyl linkage by alkaline hydrolysis before proceeding with the above estimation.

Interfering substances

The presence of any aromatic substance capable of undergoing nitration in the cold to give an acidic nitro compound will interfere in the application of this method. Phenols are also sufficiently acidic after nitration to be extracted by the 0·1 N-sodium hydroxide employed, and the bromination method described above must precede the analysis if phenols are present. With some phenols this may not be adequate, especially if abnormal amounts are present, and such specimens would not be suitable for analysis by the present method without special modification.

Among unsaturated derivatives of *cyclo*hexanecarboxylic acid, which may yield traces of aromatic acid during the reaction with the nitrating mixture, Δ^1-, Δ^2- and Δ^3-*cyclo*hexenecarboxylic acids are largely eliminated by the usual preliminary bromination treatment (Table 4). The monohydroxy*cyclo*hexanecarboxylic acids also interfere, presumably because they are first dehydrated by the sulphuric acid in the nitrating mixture and then partially aromatized and nitrated. *cyclo*Hexanecarboxylic acid itself gives no measurable amount of aromatic compound (Table 4).

Notes on the method

Nitration of benzoic acid by potassium nitrate and sulphuric acid without heat yields a mixture of mononitro derivatives, in the approximate proportion: *ortho*, 18 %; *meta*, 80 %; *para*, 2 % (cf. Houben, 1924). If *m*-nitrobenzoic acid is dissolved in the cold nitrating mixture used in stage 2 of the present method (see above) and put through the entire process it is recovered to the average extent of 89·4 %, as compared with the direct reduction of an equimolar amount of *m*-nitrobenzoic acid in stage 5 followed by colour development (stages 6 and 7). *m*-Aminobenzoic acid, after diazotization and dye-coupling, yields within 1 % the same colour as the direct reduction of *m*-nitrobenzoic acid followed by diazotization and dye-coupling. *m*-Nitrohippuric acid yields, molecule for molecule, the same colour density as *m*-nitrobenzoic acid. Benzoic or hippuric acids put through the entire process yield an average of 86 % of the colour given by direct colour develop-

Table 4. *Colorimetric equivalents (expressed as the amount of benzoic acid (mg.) required to give the same density readings) of 1 mg. quantity of various substances*

(Ilford spectrum green light filter: 1 cm. cell depth.)

Substance	Equivalent benzoic acid (mg.) Without bromination	With bromination	Colour obtained
Benzoic acid	1·0	—	Magenta
Hippuric acid	1·0	—	Magenta
Phenol	Variable	—	Deep blue
Salicylic acid	0·08	—	Purple
m-Hydroxybenzoic acid	0·20	—	Magenta
p-Hydroxybenzoic acid	0·19	—	Green changing to blue
Tyrosine	0·01	—	(Very pale)
Phenylalanine	0·01	—	(Very pale)
Benzamide	0·01	—	Faint pink
m-Nitrobenzoic acid	0·83	—	—
m-Nitrohippuric acid	0·58	—	—
*cyclo*Hexanecarboxylic acid	0·00	—	—
Hexahydrohippuric acid	0·00	—	—
Hexahydrobenzamide	0·00	—	—
Δ^1-*cyclo*Hexenecarboxylic acid	0·108	0·004	Magenta
Δ^2-*cyclo*Hexenecarboxylic acid	0·108	0·014	Magenta
Δ^3-*cyclo*Hexenecarboxylic acid	0·062	0·002	Magenta
1-Hydroxy*cyclo*hexanecarboxylic acid	0·02	—	Magenta
cis-2-Hydroxy*cyclo*hexanecarboxylic acid	0·03	—	Magenta
trans-2-Hydroxy*cyclo*hexanecarboxylic acid	0·00	—	Magenta
cis-3-Hydroxy*cyclo*hexanecarboxylic acid	0·02	—	Magenta
trans-3-Hydroxy*cyclo*hexanecarboxylic acid	0·03	—	Magenta
cis-4-Hydroxy*cyclo*hexanecarboxylic acid	0·04	0·027	Magenta
trans-4-Hydroxy*cyclo*hexanecarboxylic acid	0·03	0·023	Magenta
laevo-Quinic acid	0·00	—	—

ment from an equimolar amount of *m*-aminobenzoic acid. From these data it appears probable that there is a loss amounting only to a few per cent on nitration, and an overall loss which is nearly constant at about 14% and occurs almost entirely in the nitration and extraction stages. The extraction loss might be reduced by the use of continuous extractors, but for serial analyses the inconvenience did not appear to be justified by the possible slight gain in accuracy. *iso*Amyl alcohol was adopted for this extraction because it is a satisfactory solvent for hippuric and benzoic acids, or their mononitro compounds. If only benzoic and not hippuric acid is present, ether may be used for extraction.

A further variation which has given satisfactory recoveries in trial experiments is to hydrolyse hippuric acid present in the original sample by boiling for 2 hr. after making alkaline with excess sodium hydroxide (about 2N), extracting the benzoic acid from the acidified solution by ether or light petroleum, and evaporating the extract to dryness in presence of a slight excess of 0·1N-alkali. The dry residue is then used for the analysis as described above. In particular cases this method may be advantageous in that the alkaline boiling may remove interfering substances or hydrolyse a glycuronide, but it is generally less convenient than the normal procedure.

Reduction in acid solution of the nitro acids follows the procedure applied by Eckert (1943) to the estimation of *p*-nitrobenzoic acid; the urea used instead of ammonium sulphamate for removing excess nitrous acid appeared to be equally satisfactory. The colour-developing base chosen is that used by Bratton & Marshall (1939) for estimation of sulphonamide. The final colour obtained must be free from bluish tint and should closely match that given by alkaline dilute solutions of carmine. A bluish tint indicates either inadequate removal of nitrous acid or the presence of interfering substances, particularly phenolic compounds, in the original sample.

SUMMARY

1. A method has been developed for the microestimation of benzoic and hippuric acids in biological material. It is described for application to amounts of these acids within the range 0·05–10 mg.

2. The basis of the method is the nitration of these acids at room temperature, extraction of the resulting mononitro compounds, reduction to the corresponding amino compounds, followed by diazotization and finally coupling of the diazo compounds with a dye-forming base in acid solution. The amount of dye is closely proportional to the amount of benzoic or hippuric acid in the sample.

3. The application of the method to tissues, blood and urine is described.

We wish to thank Dr C. T. Beer for preparing a specimen of *m*-nitrohippuric acid and Miss D. Salmony for carrying out some of the estimations.

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The Aromatization of Hydrogenated Derivatives of Benzoic Acid in Animal Tissues

By C. T. BEER, F. DICKENS and JOAN PEARSON
The Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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Aromatization within the animal body is a phenomenon of special interest in view of the occurrence in many highly active biological substances of one or more aromatic rings. The origin of such compounds as the natural oestrogenic hormones, in which ring A is always aromatic and sometimes ring B also, is at present unknown. Evidence of aromatization is therefore of potential value in considering the origin of these substances within the animal body. Further, it has been suggested as a possibility that carcinogens of the type of the well known carcinogenic polycyclic aromatic hydrocarbons might arise within the animal organism as a result of a pathological metabolism of naturally occurring polycyclic hydroaromatic ring systems, such as steroids, or perhaps from the natural sex hormones by a process involving the further aromatization of these compounds.

A detailed account of these suggestions and of some related investigations, with references to the literature, has recently been given (Dickens, 1950) so that it is unnecessary to repeat it here. Some preliminary investigations were reported by Dickens (1947). In the present paper an investigation is reported into the simpler aromatization reaction as it occurs in the course of metabolism of *cyclo*hexanecarboxylic acid and its derivatives within the animal body.

The present knowledge of aromatization of the *cyclo*hexane ring, to yield benzoic acid or its derivatives, begins with the studies on the metabolism of *laevo*quinic acid (1:3:4:5-tetrahydroxy*cyclo*hexanecarboxylic acid). In an attempt, not yet fully realized, to account for the normal excretion of benzoic acid (or hippuric acid in those species which normally effect the necessary conjugation with glycine), it was found by Lautemann (1863) that in man orally administered calcium quinate was converted to hippuric acid which was isolated from the urine. Quick (1931) showed that the excretion rate was slower than that for a corresponding dose of benzoic acid, and gave similar data for the increased hippuric acid excretion following a meal of fruits (prunes) believed to contain quinic acid. Vasiliu, Timoşencu, Zaimov & Coteleu (1940) report a 54% conversion of quinic acid to benzoic acid in the sheep, but less than 6% conversion in the dog. In man, however, they obtained only 10% of the theoretical amount of benzoic acid, and this only when the diet was such that it produced an alkaline urine. In view of the discrepancy between their results and those of Lautemann and Quick, both of whom obtained considerable aromatization, we have re-investigated the fate of this acid, and have confirmed the work of Lautemann and Quick.

Apart from quinic acid, the aromatization of only a very limited number of hydroaromatic substances has hitherto been studied. Friedmann (1911) obtained some evidence of increased urinary excretion of hippuric acid in the dog after injection with *cyclo*hexanecarboxylic acid or hexahydroanthranilic acid. The latter observation has not been confirmed (Bernhard, 1938*a*), but as a result of extensive investigations by Bernhard and his co-workers it is now clear that the dog, rabbit and man can aromatize *cyclo*hexanecarboxylic acid and a limited number of closely related derivatives, e.g. hexahydro-*N*-methylbenzamide, hexahydrohippuric acid, *cyclo*hexylvaleric acid (Bernhard, 1937, 1938*a*, *b*; Bernhard & Caflisch-Weill, 1945). The related literature has recently been fully reviewed (Dickens, 1950). It is only necessary, therefore, to mention here that by the use of deuterium-labelled *cyclo*hexanecarboxylic acid, Bernhard & Caflisch-Weill (1945) were able to show that the source of the urinary benzoic acid was, in fact, the administered *cyclo*hexane compound.

The object of the present investigation was twofold: (*a*) to obtain information on the chemical route and enzymic nature of this transformation of the *cyclo*hexane to the benzenoid ring, (*b*) to obtain aromatization in isolated tissues, if possible; since this is the only practicable method of excluding the participation of intestinal flora in the reaction and of ascertaining the probable site at which it occurs in the animal body.

EXPERIMENTAL

Materials and methods

cyclo*Hexane- and* cyclo*hexene-carboxylic acids.* *cyclo*Hexanecarboxylic acid and *cyclo*hexylacetic acid were purchased (British Drug Houses Ltd.). The following compounds were synthesized as in the references cited: Δ^1- and Δ^2-*cyclo*hexenecarboxylic acids and *cis*-3-hydroxy*cyclo*hexanecarboxylic acid (Boorman & Linstead, 1935); 1-hydroxy*cyclo*hexanecarboxylic acid (Bucherer, 1894); *trans-*

2-hydroxy*cyclo*hexanecarboxylic acid (Mousseron, Jullien & Winternitz, 1948); *trans*-3-hydroxy*cyclo*hexanecarboxylic acid (Perkin & Tattersall, 1907); *cis*-4-hydroxy*cyclo*hexanecarboxylic acid (Hardegger, Plattner & Blank, 1944).

The preparation and nature of *trans*-4-hydroxy*cyclo*hexanecarboxylic acid calls for special comment. The earlier experiments of Beer, Dickens & Pearson (1949, see also Dickens, 1950), were made with a specimen of the so-called *trans* isomer of this acid prepared according to Perkin (1904), m.p. 120–121°. When this work was nearly completed Campbell & Hunt (1950) showed that this was in fact a *cis-trans* mixture, the true *trans* isomer having m.p. 148°. We are greatly indebted to these authors for specimens of the *cis* and *trans* isomers of 4-hydroxy*cyclo*hexanecarboxylic acid with which we have repeated our earlier experiments. Only the results on the pure *trans* isomer are reported here.

cis-2-Hydroxy*cyclo*hexanecarboxylic acid was prepared by the method of Pascual, Sistaré & Regás (1949), except that the use of Raney nickel (prepared according to Delépine & Horeau, 1937) instead of platinic oxide shortened the time required for the catalytic reduction of the intermediate ethyl *cyclo*hexan-2-one-1-carboxylate to approximately 90 min. at atmospheric temperature and pressure.

Δ^3-*cyclo*Hexenecarboxylic acid: Butadiene (5 g.), methyl acrylate (12 g.) and hydroquinone (0·05 g.) were heated in a sealed tube at 130° for 10 hr. When cold the contents of the tube were diluted with ether (100 ml.), washed with 2N-NaOH and water, and dried (Na_2SO_4). Distillation gave methyl Δ^3-*cyclo*hexenecarboxylate (12·5 g.) as a clear colourless oil b.p. 69–71°/15 mm. The latter (12 g.) was refluxed for 2 hr. with a mixture of KOH (15 g.), ethanol (60 ml.) and water (10 ml.). After removing the ethanol in a current of steam, the alkaline solution was washed with ether, acidified with 5N-HCl and continuously extracted (12 hr.) with ether in an atmosphere of N_2. The ethereal extract was dried (Na_2SO_4) and distilled, giving Δ^3-*cyclo*hexenecarboxylic acid as a colourless oil (9·75 g.) b.p. 120–122°/10 mm., which rapidly crystallized on cooling, m.p. 18–18·5°. (Sobecki (1910) gives m.p. 13–15°.) (Found: C, 66·55; H, 8·35. Calc. for $C_7H_{10}O_2$: C, 66·6; H, 8·0%.) The acid, in cold $CHCl_3$ solution, rapidly absorbed the theoretical quantity of Br_2 required to saturate one double bond, no HBr being eliminated. After removal of the solvent the dibromide, crystallized from formic acid, had m.p. 85–86° (Perkin & Tattersall, 1907, m.p. 86°).

Where optical isomerides could exist all the above compounds were used as the racemic forms.

Estimation. Benzoic (or hippuric) acid was estimated by the colorimetric method of Dickens & Pearson (1950). All results are expressed as benzoic acid regardless of whether benzoic or hippuric acid was actually present in the samples. Details of any special preliminary treatment required to prepare urine or tissue extracts for analysis are mentioned in the appropriate sections below.

Diet and dosage. Rabbits of 2–3 kg. were maintained in metabolism cages on a diet of cabbage *ad lib.* The compounds were usually injected subcutaneously as single doses of about 10 ml. of the neutral aqueous solutions of their Na salts; in a few instances the Na salt was given by mouth. No toxic effects were observed at any of the dose levels employed, although occasionally there was a marked decrease in the volume of urine excreted during the 24 hr. following injection. The urine was, however, of usual appearance and pH.

Collection and estimation. (*a*) *Urine.* A control urine sample was collected during the 24 hr. period preceding injection and usually for two subsequent 24 hr. periods. No preservative was used since no aromatization was observed when selected compounds were dissolved in normal urine and kept 24 hr. Similarly, benzoic acid added to urine was recovered undiminished after standing for 24 hr. Estimations were made on 5 ml. portions of the urine adjusted to pH 6·8 with HCl and filtered through gauze. The filtrate was treated with 5 ml. saturated Br_2 water and allowed to stand for 30 min. to precipitate phenolic compounds and saturate the double bonds of *cyclo*hexene derivatives. After filtration, excess Br_2 was removed from the solution by the passage of a stream of N_2. Portions of 1 ml. each were diluted to 10 ml. with 0·1N-HCl, extracted with *iso*amyl alcohol, and the estimation completed as described by Dickens & Pearson (1950).

The values of urinary benzoic acid excretion given in this paper are corrected for the basal figures obtained during the 24 hr. preceding the administration of the dose. In rabbits fed solely on cabbage this basal value is favourably low, the mean on sixteen rabbits being 24 mg./24 hr. (cf. Table 1, column '−24 to 0 hr.').

(*b*) *Blood.* The benzoic or hippuric acid in whole blood was determined colorimetrically on a 3·5 or 5 ml. sample collected from the ear vein of the rabbit (Dickens & Pearson, 1950).

Isolation of hippuric and benzoic acids from urine. Two methods were used. (i) The neutralized urine was evaporated to a syrup and the extraction and crystallization of hippuric acid followed as described by Friedmann (1911). (ii) The urine was acidified to congo red with HCl and continuously extracted with ether for 14 hr. Benzoic acid was readily isolated in this way, but hippuric acid, if present in the urine, is liable to become hydrolysed to benzoic acid and glycine during the extraction.

Tissue slices. Tissue slices (usually 300 mg. wet wt.) were incubated in O_2 at 37° in Warburg manometer vessels containing 2 ml. Krebs's Ringer solution, buffered with phosphate. In some experiments the gas was O_2 containing 5% CO_2 and the buffer used was then bicarbonate. Substrates were added as neutral solutions of their Na salts. At the end of the incubation period (usually 4 hr.), the pH was checked (6·8–7·2), and the whole contents of the vessels (after removal of the KOH used for absorption of CO_2) were then diluted to 15 ml. with 70% (v/v) ethanol. Benzoic acid was estimated colorimetrically in measured portions of the filtrate (Dickens & Pearson, 1950). Vessels from which the tissue was omitted were simultaneously incubated to provide a reagent blank and to reveal any tendency of the compounds to autoxidize to substances estimable as benzoic acid: this correction was always very small under the conditions employed.

Tissue homogenates. (*a*) *Preparation.* No success followed attempts to prepare active fatty acid oxidizing homogenates from rat or rabbit liver with a Waring type blender after the general procedure of Green, Loomis & Auerbach (1948). These authors, however, worked with much larger quantities than were used in the present work, and the enzyme system was evidently unstable under the conditions used by us.

By using a glass homogenizer (Potter & Elvehjem, 1936) and following a standardized procedure, no difficulty was experienced in preparing homogenates able actively to oxidize *n*-octanoic acid in a reproducible manner. The conditions finally adopted were as follows. The animal (rabbit or rat) was killed by a blow on the head, quickly bled and the liver immediately removed into crushed ice. Portions of 1·5 g. liver were weighed into 5 ml. ice-cold 0·15M-KCl, and

homogenized with a loosely fitting glass pestle for 20 sec. at 0°. A further 7·5 ml. ice-cold 0·15 M-KCl and 1 ml. 0·1 N-KOH were then added and homogenizing was resumed for a further 100 sec. The 10% homogenate so obtained had pH 7·0–7·4 and was readily pipetted. It was always prepared immediately before use, being the last component (1 ml.) to be added to the vessels before filling them with O_2.

(*b*) *Measurement of respiration.* The final concentrations in 3 ml. diluted homogenate contained in each Warburg vessel were: phosphate buffer pH 7·4, 0·015 M; $MgCl_2$, 0·013 M; cytochrome *c*, 10^{-5} M; adenosinetriphosphate, 0·001 M. The substrates were added as neutral solutions of their Na salts; final pH 7·0–7·2. Each vessel had homogenate equivalent to 100 mg. wet tissue. The flasks were gassed with O_2 at room temperature for 2 min., immediately placed in the bath at 37° and readings begun after 5 min. The oxidative activity of the homogenate was compared with that towards 0·001 M-*n*-octanoate in each experiment. Colorimetric benzoic acid determinations were made on the vessel contents by the method already described for tissue slices.

RESULTS

Aromatization in the intact animal

Urinary excretion in the rabbit. Table 1 summarizes the observations on the urinary excretion of benzoic or hippuric acids after the subcutaneous injection into rabbits of the compounds listed. Control experiments using benzoic acid showed that some 70% of the compound was recoverable in the urine as hippuric acid, elimination being virtually complete within 24 hr. (Table 1). This agrees well with the findings of Bray, Neale & Thorpe (1946).

Where an appreciable amount of benzoic acid was indicated by the colorimetric method of estimation, the aromatic acid responsible was isolated with the sole exception of Δ^1-*cyclo*hexenecarboxylic acid where isolation was not carried out. In all cases the product was benzoic or hippuric acid, and the

Table 1. *Benzoic acid excretion in the urine of rabbits receiving subcutaneous injections of* cyclo*hexane-*, cyclo*hexene- and monohydroxy*cyclo*hexane-carboxylic acid (as sodium salts)*

Compound	Rabbit wt. (kg.)	Dose* (mg.)	Benzoic acid* excretion (mg.) during hr. −24 to 0	0 to 24	24 to 48	Method†	Extra benzoic* acid (mg.)	Extra benzoic as percentage of dose
Benzoic acid	2·42	1750	16·5	1346	28·2	*c*	1341·2	76·7
Benzoic acid	2·47	1640	37·7	1150	N.D.	*c*	1112·3	67·8
*cyclo*Hexanecarboxylic acid	1·91	1888	28·4	947	145·6	*c*	1035·8	55·0
*cyclo*Hexanecarboxylic acid	2·51	1077	18·3	550	35·7	*c*	549·1	51·0
*cyclo*Hexanecarboxylic acid	2·70	493	39·2	248·2	16·5	*c*	186·3	37·8
*cyclo*Hexanecarboxylic acid	N.D.	1300	N.D.	650		*i*	—	50 (approx.)
Δ^1-*cyclo*Hexanecarboxylic acid	2·75	1642	28·0	1018	176	*c*	1138	69·3
Δ^2-*cyclo*Hexenecarboxylic acid	2·67	1840	90·0	755	84·1	*c*	659·1	35·8
			—	669		*i*	—	32 (approx.)
Δ^3-*cyclo*Hexenecarboxylic acid	2·87	1936	32·7	1020	111	*c*	1065·6	55·1
			—	1155		*i*	—	60 (approx.)
Δ^3-*cyclo*Hexenecarboxylic acid	1·84	1788	4·8	512·5	56·5	*c*	559·4	31·3
Δ^3-*cyclo*Hexenecarboxylic acid	3·08	2015	—	675		*i*	—	33 (approx.)
1-Hydroxy*cyclo*hexane-carboxylic acid	1·92	846	19·0	43·4	N.D.	*c*	24·4	2·9
cis-2-Hydroxy*cyclo*hexane-carboxylic acid	3·12	1580	4·7	47·3	N.D.	*c*	42·6	2·7
trans-2-Hydroxy*cyclo*-hexanecarboxylic acid	2·22	1690	10·4	52·9	N.D.	*c*	42·5	2·5
cis-3-Hydroxy*cyclo*hexane-carboxylic acid	2·50	1780	25·4	49·7	0·0	*c*	24·3	1·4
trans-3-Hydroxy*cyclo*-hexanecarboxylic acid	2·50	1830	13·6	67·7	N.D.	*c*	54·1	3·0
cis-4-Hydroxy*cyclo*hexane-carboxylic acid	2·73	410	5·3	9·4	N.D.	*c*	4·1	1·0
trans-4-Hydroxy*cyclo*-hexanecarboxylic acid	2·50	770	20·4	62·7	68·2	*c*	90·1	11·7

N.D. = not determined.

* Doses and amounts of excreted hippuric acid are both expressed as the equivalent amounts of benzoic acid.

† Method: *c* = Colorimetric; *i* = isolation as hippuric acid. (Blanks are not subtracted in isolation experiments, which covered the period 0–48 hr.)

amount isolated corresponded with that found colorimetrically. Analysis of urine samples passed during the first and second 24 hr. periods showed that the excretion of the benzoic acid was usually virtually complete within 24 hr., in agreement with the analyses of blood benzoic acid described below which show that the aromatization is a comparatively rapid process. Fig. 1 shows the relationship of the amounts of benzoic acid excreted to the doses of *cyclo*hexane compound injected.

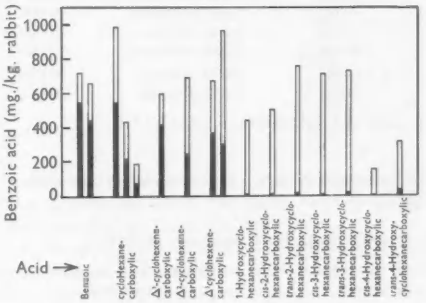

Fig. 1. Excretion of benzoic acid by rabbits following the subcutaneous injection of hydroaromatic acids. Total height of columns = weight of compound injected/kg. rabbit (expressed as the theoretically corresponding amount of benzoic acid); solid portion of column = weight of extra benzoic acid found in urine.

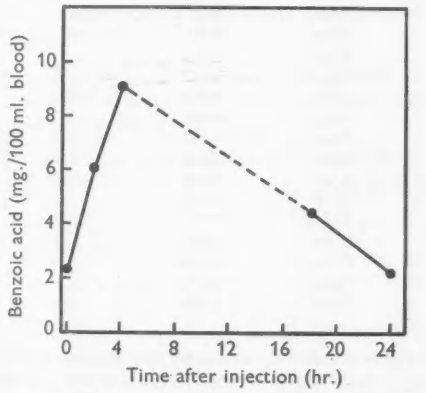

Fig. 2. Level of benzoic acid in rabbit blood after subcutaneous injection of *cyclo*hexanecarboxylic acid. Dose equivalent to 0·97 g. benzoic acid.

A slight fall was observed in the percentage conversion to benzoic acid excreted as the dose level (mg./kg.) of *cyclo*hexanecarboxylic acid was reduced (cf. Fig. 1). In considering the remaining compounds a striking difference is at once apparent between the hydroxy*cyclo*hexanecarboxylic acids, none of which was much aromatized, and the three *cyclo*hexenecarboxylic acids, all of which yielded large quantities of benzoic acid. The proportion of the latter excreted following the injection of the Δ^1 compound (69 %) approaches very nearly that obtained by the direct administration of benzoic acid itself (67–76 %). Δ^2- And Δ^3-*cyclo*hexenecarboxylic acids appear to be rather less readily converted to benzoic acid than the Δ^1 compound in the whole rabbit.

Blood benzoic acid in the rabbit. Fig. 2 shows the increase of benzoic acid content of the blood following the subcutaneous injection into a 3·05 kg. rabbit of an aqueous solution containing *cyclo*hexanecarboxylic acid (as sodium salt) equivalent to 0·97 g. benzoic acid. During the first 4 hr. after injection the blood benzoic acid rose rapidly, but 18–24 hr. after injection it had returned to a low level.

Urinary excretion in the rat. Twelve rats in metabolism cages were injected at 0 hr., after collection of urine for the preceding 24 hr., with a total amount of 24 ml. solution of sodium *cyclo*hexanecarboxylate, equivalent to 2·42 g. benzoic acid. The urine was collected over the periods shown in Table 2 in which the colorimetrically estimated amounts of benzoic acid are shown. The residue from evaporation of the ether extracts was crystallized from hot water, but only minimal amounts of benzoic acid were found (Table 2).

Table 2. *Urinary excretion of benzoic acid by twelve rats after subcutaneous injection of sodium* cyclo*hexanecarboxylate*

(Total dose equivalent to 2·42 g. benzoic acid.)

Period (hr.)	Vol. urine (ml.)	Benzoic acid excretion (mg.)	Benzoic acid excretion Percentage of dose
−24 to 0	157	15·5	—
0 to 24	235	109·4*	3·9
24 to 48	144	30·9	0·6

* By ether extraction, hydrolysis with NaOH and recrystallization, benzoic acid, m.p. 121°, was identified.

Aromatization in isolated tissues

Tissue slices with cyclo*hexanecarboxylic acid as substrate*

Ability of various tissues to aromatize cyclo*hexanecarboxylic acid.* Both liver and kidney slices of rabbits and rats were found to produce benzoic acid when incubated with *cyclo*hexanecarboxylic acid (Table 3). In both species liver slices were more efficient than kidney slices and the rabbit tissues were more active than the corresponding rat tissues in effecting the aromatization. None of the other rabbit tissues tested (Table 3) was found to yield other than small quantities of benzoic acid. (The

Table 3. *Formation of benzoic acid from* cyclo*hexanecarboxylic acid by slices of various tissues*

(Incubation at 37° in O_2; slices suspended in phosphate-Ringer solution.)

Animal	Tissue	Tissue wt. (mg.)	Incubation time (hr.)	Benzoic acid found (mg.) Tissue only	Tissue + *cyclo*hexane-carboxylate (concn.)	Formed
Rat 1	Liver	300	3	0·00	0·07 (0·005 M)	0·07
Rat 2	Kidney	200	2	0·026	0·040 (0·025 M)	0·014
Rabbit 1	Liver*	300	3	0·034	0·120 (0·004 M)	0·086
	Testis	300	3	0·034	0·034 (0·004 M)	0·00
	Spleen	300	3	0·081	0·082 (0·004 M)	0·001
	Skeletal muscle (mince)	400	3	0·050	0·055 (0·01 M)	0·005
	Blood (defibrinated)	(10 ml.)	3	0·203	0·224 (0·004 M)	0·021
Rabbit 2	Kidney	200	3	0·061	0·099 (0·05 M)	0·038
		200	1·5	0·061	0·083 (0·05 M)	0·022

* For further experiments on *cyclo*hexanecarboxylic acid with rabbit liver see Tables 4–6.

Table 4. *Effect of incubation conditions on benzoic acid formation from* cyclo*hexanecarboxylic acid by rabbit-liver slices*

(Concn. of HCN, 10^{-3} M. Incubation period, 3 hr. P = phosphate, B = bicarbonate medium.)

Rabbit no.	Medium	*cyclo*Hexane-carboxylate concn. (M)	Gas phase	Inhibitor	Benzoic acid at end of experiment Total (mg.)	Extra due to substrate (mg.)
1	P	0·00	O_2	None	0·03	—
		0·02	O_2	None	0·15	0·12
		0·004	O_2	None	0·19	0·16
		0·001	O_2	None	0·21	0·18
		0·00	N_2	None	0·05	—
		0·002	N_2	None	0·06	0·01
		0·001	N_2	None	0·05	0·00
2	P	0·00	O_2	None	0·034	—
		0·04	O_2	None	0·120	0·086
		0·04	O_2	HCN	0·064	0·030
3	P	0·00	O_2	None	0·030	—
		0·01	O_2	None	0·163	0·133
3	B	0·00	O_2+CO_2	None	0·028	—
		0·01	O_2+CO_2	None	0·228	0·200
		0·00	O_2+CO_2	HCN	0·022	—
		0·01	O_2+CO_2	HCN	0·070	0·048
4	P	0·00	O_2	None	0·025	—
		0·002	O_2	None	0·159	0·134
4	B	0·00	O_2+CO_2	None	0·023	—
		0·002	O_2+CO_2	None	0·229	0·206

value recorded in Table 3 for blood is doubtful as the final colour was atypical.)

This preliminary survey suggested that rabbit liver was the most suitable tissue with which to study the aromatization reaction, and before extending the investigation to the unsaturated and hydroxy acids an examination was made of the factors which might influence the formation of benzoic acid by rabbit-liver slices from *cyclo*hexanecarboxylic acid.

Effect of incubation conditions. Cyanide powerfully inhibited the formation of benzoic acid by rabbit-liver slices from *cyclo*hexanecarboxylic acid. The replacement of oxygen by nitrogen in the incubation vessel also prevented aromatization (Table 4). Under aerobic conditions the yield of benzoic acid was slightly higher in a bicarbonate than in a phosphate-buffered Ringer solution (Table 4).

Extent of benzoic acid formation from cyclo*hexanecarboxylic acid by liver slices from different rabbits.* It became apparent early in this work that the livers from different healthy rabbits varied considerably in their ability to produce benzoic acid from *cyclo*hexanecarboxylic acid. As seen in Table 5, the benzoic acid produced in 4 hr. by 300 mg. of rabbit

Table 5. *Production of benzoic acid from* cyclo*hexanecarboxylic acid by rabbit-liver slices*

(Warburg vessels contained: 300 mg. liver slices; 0·1 M-phosphate Ringer solution and *cyclo*hexanecarboxylate of stated molarity. Final volume of liquid 2 ml.; incubated at 37° in O_2. *a* And *b* here and in Tables 6 and 7 are estimations on different portions of slices from the same liver.)

Liver no.	Substrate concn. (M)	Incubation time (hr.)	Benzoic acid formed (mg.) (*a*)	(*b*)
1	0·01	4	0·096	0·10
2	0·002	4	0·077	0·085
3	0·002	4	0·130	0·164
4	0·01	4	0·137	0·127
5	0·01	4	0·08	
	0·01	8	0·09	0·123
6	0·01	4	0·151	0·202
	0·005	4	0·154	0·225
7	0·01	4	0·135	0·135

liver slices varied from 0·077 to 0·225 mg. in different livers; there is some variation between different portions of slices from the same liver.

Effect of substrate concentration on the production of benzoic acid by liver slices. The results in Table 6 show that the production of benzoic acid from *cyclo*hexanecarboxylic acid is nearly independent of substrate concentration between 0·025 and 0·005 M. At 0·05 M and above, some decline in degree of aromatization was found. The respiration rate of the slices was not noticeably affected by the addition of substrate up to a concentration of 0·1 M. There was no autoxidation of *cyclo*hexanecarboxylic acid incubated without tissue, nor was any benzoic acid found (Table 6).

Table 6. *Effect of substrate concentration on the aromatization of* cyclo*hexanecarboxylic acid by rabbit-liver slices*

(Conditions as in Table 5; incubation time 4 hr.)

Substrate concentration (M)	Tissue absent	Benzoic acid found (mg.) Tissue present (*a*)	(*b*)
0·0	—	0·00	0·02
0·1	0·01	—	—
0·05	—	0·14	0·14
0·025	—	0·22	0·18
0·0125	0·00	0·24	0·13
0·01	—	0·21	0·16
0·005	0·01	0·24	0·17

Rate of formation of benzoic acid by tissue slices. Although the formation of benzoic acid from *cyclo*hexanecarboxylic acid by liver slices has been shown to require active respiration, absence of increased respiration in the presence of substrate does not suggest a close connexion between aromatization and the extent of oxygen consumption by the tissue. The oxygen consumption and benzoic acid formation in presence of *cyclo*hexanecarboxylic acid were therefore determined at intervals during incubation (Fig. 3). It was found that the rate of benzoic acid formation fell off rapidly after 2 hr., whereas the oxygen consumption of the tissue fell only slightly compared with the control.

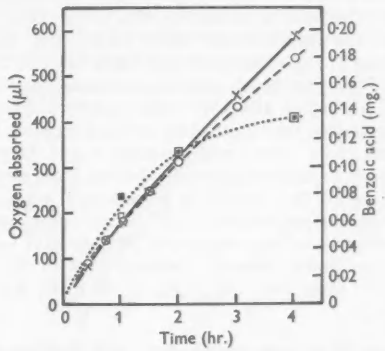

Fig. 3. Rate of formation of benzoic acid in relation to rabbit-liver slice respiration. 300 mg. slices in 2 ml. 0·1 M-phosphate buffer (37° in O_2). ×—×, O_2 absorption, tissue only; ○– – –○, O_2 absorption, tissue + 0·005 M-*cyclo*hexanecarboxylate; ....., extra benzoic acid formed in presence of *cyclo*hexanecarboxylate (□ and ■, duplicate experiments).

Isolation of hippuric acid formed from cyclo*hexanecarboxylic acid by rabbit-liver slices.* The liver of a freshly killed rabbit (wt. 2·8 kg.) was rapidly sliced, connective tissue (5 g.) being discarded. The material was equally divided between two flasks (42 g. in each) and rinsed in 100 ml. bicarbonate-Ringer solution while aerated with oxygen containing 5% carbon dioxide. The rinsing medium in both flasks was replaced by 430 ml. of fresh buffer, and to one of the flasks was added 0·5 ml. 1 M-

sodium *cyclo*hexanecarboxylate. Oxygen-carbon dioxide mixture was passed through the flasks whilst the latter were gently rocked at 37° for 3 hr. After cooling, the contents of the flasks were treated with 98 % ethanol (645 ml.), stood 1 hr., and filtered.

Colorimetric benzoic acid determinations made on samples (10 ml.) from each of the 1075 ml. filtrates thus obtained, gave the figures: *cyclo*hexanecarboxylic acid absent, 1·1 mg.; *cyclo*hexanecarboxylic acid present, 33·4 mg.; extra benzoic acid found, 32·2 mg. in the total filtrate.

The tissue retained on the filter was washed with 60 % aqueous ethanol (100 ml.), and the latter combined with the portion of the appropriate filtrate remaining after the foregoing colorimetric estimations. After evaporation *in vacuo* to about 230 ml. each of the concentrates was treated with 8 ml. 2N-hydrochloric acid and 1·5 g. sodium tungstate in 3 ml. water. After 1 hr. the precipitated material was removed by filtration and washed with 20 ml. water. The combined filtrates were adjusted to pH 8·5 with approximately 5 ml. 2N-sodium hydroxide and concentrated *in vacuo* to about 50 ml. After acidification with 2·5 ml. concentrated hydrochloric acid, the solutions were extracted continuously with ether for 24 hr. The residue remaining after evaporating the ether was recrystallized from 2·5 ml. hot water, yielding 21 mg. of hippuric acid, m.p. 184–185°. The mixed melting point with an authentic specimen (m.p. 187°) was 186–187°. The *p*-nitrobenzyl ester was prepared by refluxing the isolated hippuric acid (20 mg.) for 1 hr. with *p*-nitrobenzyl bromide (35 mg.), N-sodium hydroxide (0·12 ml.) and ethanol (0·35 ml.). On cooling *p*-nitrobenzyl hippurate (21 mg.) crystallized, m.p. 131°, raised after one recrystallization from ethanol to 135°, alone or mixed with authentic material. (Found: C, 61·6, H, 4·4; N, 8·2. Calc. for $C_{16}H_{14}O_5N_2$: C, 61·1; H, 4·5; N, 8·9 %.)

Tissue slices with cyclo*hexene- and hydroxy*cyclo*hexane-carboxylic acids as substrates*

The marked difference in the capacity of various rabbit livers to aromatize *cyclo*hexanecarboxylic acid has already been mentioned. In comparing, therefore, the ability of other substrates to act as precursors of benzoic acid the several compounds were either used as substrates for slices from the same liver or, when working with livers from different animals, *cyclo*hexanecarboxylic acid was used as a common reference substance. The amounts of benzoic acid obtained with the three *cyclo*hexenecarboxylic acids as substrates (0·01 M) are shown in Fig. 4. Whilst Δ^1-*cyclo*hexenecarboxylic acid was aromatized to barely half the extent of *cyclo*hexanecarboxylic acid, the Δ^3 compound produced almost double the amount of benzoic acid given by that substance. The Δ^2 acid yielded about the same amount of benzoic acid as the *cyclo*hexanecarboxylic acid control.

A number of the hydroxy*cyclo*hexanecarboxylic acids was also studied as substrates for aromatization by liver slices. Some reserve is necessary in interpreting the results of the colorimetric benzoic acid estimations in media containing compounds of this class, since they alone gave quite appreciable blank values for benzoic acid. This is probably due to the formation of a *cyclo*hexenecarboxylic acid as a result of the dehydrating action of the sulphuric acid during the nitration stage. The unsaturated acids

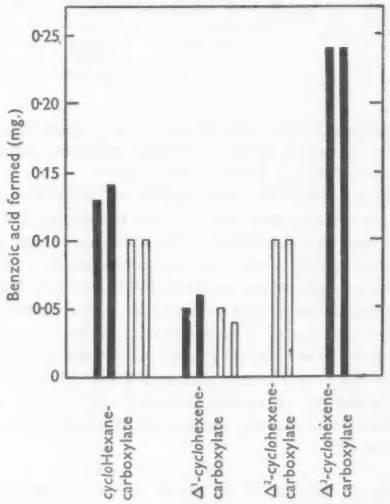

Fig. 4. Aromatization of *cyclo*hexane- and *cyclo*hexenecarboxylic acids by rabbit-liver slices. 300 mg. slices incubated 4 hr. in 2 ml. 0·1 M-phosphate buffer (at 37° in O_2). Substrate concn. 0·01 M. Open columns, rabbit 1; solid columns, rabbit 2.

have been shown (Dickens & Pearson, 1950) to return appreciable values for benzoic acid in this method unless they are saturated by bromination, and they would therefore be expected to yield some substance which is estimated as benzoic acid under the above conditions. In fact, during the incubation with liver slices the amount of some of the added hydroxy compounds appeared to diminish, presumably by oxidative breakdown (see below). The result of this would be to cause a fall in the 'blank' value due to the hydroxy compound; and this is presumably the reason for the negative values of 'benzoic acid' reported in Table 7. This table, summarizing the results obtained on incubating the hydroxy compounds with rabbit-liver slices, shows, however, that even if the compound blank is ignored entirely, the benzoic acid values are not much

Table 7. *Hydroxycyclohexanecarboxylic acid as substrates for respiring rabbit-liver slices*

(Conditions as in Table 5. Incubation time, 4 hr.; period of measurement of respiration also 4 hr. except where otherwise stated.)

Substrate			O_2 absorbed (μl.)		Benzoic acid found (mg.)		
						Compound present	
Position of substituent		Conen. (M)	Compound absent	Compound present	Compound absent	Uncorrected	Corrected*
1-OH		0·008	224	208	0·076	0·068	0·018
		0·005	—	—	0·076	0·090	0·060
cis-2-OH		0·010	576	550	0·016	0·019	0·019
		0·005	576	602	0·016	0·006	0·006
cis-3-OH		0·010	288†	255†	0·006	0·057	0·007
		0·008	288†	254†	0·006	0·061	0·000
cis-4-OH	(*a*)	0·030	772	787	0·000	0·135	−0·226
		0·010	772	730	0·000	0·048	−0·049
		0·005	772	756	0·000	0·025	−0·029
	(*b*)	0·008	248	342	0·008	0·057	−0·024
		0·005	248	293	0·008	0·035	−0·013
trans-4-OH		0·030	772	1000	0·000	0·206	0·016
		0·010	772	850	0·000	0·074	0·009
		0·005	772	830	0·000	0·050	0·016

* Corrected for amount of 'benzoic acid' as estimated on the quantity of substrate originally present in the vessels (see text).

† O_2 uptake for first 155 min. only.

greater than those in the control without substrate, and after subtraction of the analytical correction for the compound blank they are generally lower than the control and may even be negative as explained above.

Also included in Table 7 are some values for the oxygen consumption of the liver slices with and without the hydroxy compounds as substrates. Further estimations (not included in Table 7) showed that whilst 1-hydroxy-, *cis*-2-hydroxy- and *cis*-3-hydroxy-*cyclo*hexanecarboxylic acids had no significant effect, an appreciable increase in respiration was sometimes observed when the substrate was *cis*- or *trans*-4-hydroxy*cyclo*hexanecarboxylic acid (see Table 7).

Tissue homogenates

Rat-liver homogenate. In the initial experiments rat-liver homogenates were used in order to find if a system able rapidly to oxidize fatty acids such as *n*-octanoic could also utilize the *cyclo*hexane- and *cyclo*hexene-carboxylic acids as substrates, effecting thereby their aromatization to benzoic acid. The advantage to be gained by working with a homogenate instead of liver slices is clear from Fig. 5 which shows the relatively small increase in the respiration rate of slices with added *n*-octanoate in comparison with the homogenate. In these experiments the primary intention was to find an enzyme system capable of aromatizing *cyclo*hexane derivatives, and therefore no attempt was made to purify the crude homogenate as was done, for example, in the cyclophorase or mitochondrial fatty acid oxidizing preparations used by Grafflin & Green (1948) and Schneider (1948). Such purification might have involved the loss of a component of the system which oxidizes the *cyclo*hexane derivatives.

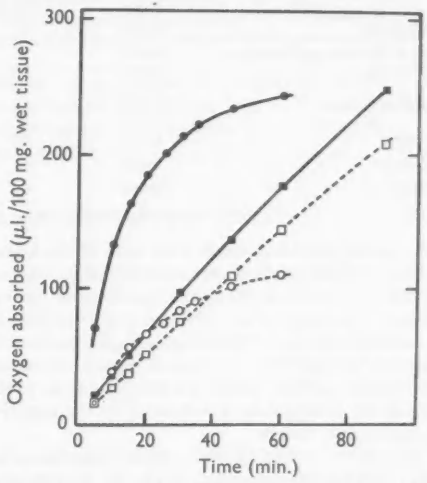

Fig. 5. Time course of oxidation of *n*-octanoate by rat-liver slices and homogenate. Slices: 300 mg. in 2 ml. 0·1 M-phosphate buffer (at 37° in O_2), □- - -□, tissue only; ■—■, tissue + 0·001 M-*n*-octanoate; O_2 uptake plotted for 100 mg. wet tissue. Homogenate: conditions as described in 'Methods', ○- - -○, homogenate only; ●—●, homogenate + 0·001 M-*n*-octanoate.

However, even the crude rat-liver homogenates, though their oxidizing activity towards normal

Table 8. *Oxygen uptake and benzoic acid formation in rat-liver homogenates in presence of various substrates*

(Experimental details as described under 'Methods'.)

		Oxygen (μl.) absorbed in 30 min.				
A	*B*	*C*	*D*	*E*	*F*	*G*
Acid	Concn. (M)	Tissue only	+ *n*-octanoate (0·001 M)	+ compound as in columns *A* and *B*	+ compound + *n*-octanoate (0·001 M)	Benzoic acid formed (columns *E–C*, mg.)
None	—	73 (av.)	192 (av.)	—	—	—
Benzoic	0·001	95	229	62	197	—
*cyclo*Hexanecarboxylic	0·001	77	190	48	160	0·00
	0·003	77	190	57	117	N.D.
Hexahydrohippuric	0·001	95	229	69	203	0·00
Δ¹-*cyclo*Hexenecarboxylic	0·001	69	205	54	202	0·00
Δ²-*cyclo*Hexenecarboxylic	0·001	69	160	53	163	N.D.
	0·003	(80)	198	53	N.D.	0·017
Δ³-*cyclo*Hexenecarboxylic	0·003	(83)	210	87	116	0·00
1-Hydroxy*cyclo*hexane-carboxylic	0·001	49	185	39	178	N.D.
cis-2-Hydroxy*cyclo*hexane-carboxylic	0·003	80	202	44	195	0·00
trans-2-Hydroxy*cyclo*hexane-carboxylic	0·003	80	202	69	204	0·00
cis-3-Hydroxy*cyclo*hexane-carboxylic	0·003	90	206	80	189	N.D.
trans-3-Hydroxy*cyclo*hexane-carboxylic	0·003	90	206	80	206	N.D.
cis-4-Hydroxy*cyclo*hexane-carboxylic	0·003	78	203	103	N.D.	−0·030
	0·010	78	203	124	244	−0·065
trans-4-Hydroxy*cyclo*hexane-carboxylic	0·003	78	203	72	N.D.	N.D.
	0·010	78	203	71	195	−0·045
*cyclo*Hexylacetic	0·001	77	199	54	84	N.D.
	0·003	77	199	52	69	0·0
Palmitic	0·001	60	156	78	N.D.	N.D.
n-Butyric	0·005	65	204	103	226	N.D.
*iso*Butyric	0·005	65	204	53	199	N.D.

N.D. = Not determined; values in parentheses are estimated by average.

fatty acids was comparable with that of the above authors' preparations, showed no comparable degree of activity in aromatizing the *cyclo*hexane derivatives. In fact benzoic acid, if found at all, was present in amounts too low to be estimated in the incubation mixture with all the compounds tested, which are included in Table 8. Again the disappearance of the 4-hydroxy compounds is indicated by the negative 'benzoic acid' values.

The effect of *cyclo*hexane-, hydroxy*cyclo*hexane- and *cyclo*hexene-carboxylic acids on homogenate respiration is also shown in Table 8. The fatty acid oxidizing capacity has been taken to be measured by the increased oxygen consumption in the presence of *n*-octanoic acid (columns *D–C*, Table 8).

Most of the compounds were found to depress endogenous respiration of the homogenate without markedly interfering with the extra oxygen uptake in the presence of *n*-octanoic acid. Octanoate oxidation, however, was strongly depressed in presence of *cyclo*hexanecarboxylic acid and Δ³-*cyclo*hexenecarboxylic acid (0·003 M). *cyclo*Hexylacetic acid is an even stronger inhibitor and in 0·001 and 0·003 M concentration depressed oxygen uptake by 58 and 65 % respectively. Since it had much less effect on the liver respiration in absence of octanoate (depression 30–32 %), it is evidently a specific inhibitor of fatty acid oxidation (cf. inhibitory effect of 2-phenylbutyric acid mentioned by Grafflin & Green, 1948).

On the other hand *cis*- but not *trans*-4-hydroxy-*cyclo*hexanecarboxylic acid, particularly in higher concentration, increased the respiration of rat-liver homogenate in absence of *n*-octanoate, and the *cis* isomer also increased the total oxygen uptake of the homogenate in presence of *n*-octanoate (Table 8).

Previous workers (Grafflin & Green, 1948) have found with fairly crude kidney homogenates that the branching of the alkyl chain of a fatty acid frequently hinders oxidation beyond the substituted

carbon atom, which they considered led to the accumulation of α- or β-alkyl fatty acids. Liver homogenates unless too far purified were, however, able to oxidize branched-chain acids (e.g. *iso*caproic and *iso*butyric) to carbon dioxide and water (Grafflin & Green, 1948, footnote p. 106).

In the present work the straight-chain fatty acids, *n*-butyric and palmitic, both increased the respiration of the rat-liver homogenate (Table 8). On the other hand, 0·005 M-*iso*butyric acid (which may be considered to have a structural resemblance to *cyclo*hexanecarboxylic acid) was apparently not oxidized by our preparation, being perhaps slightly inhibitory to oxidation with or without added *n*-octanoic acid.

Rabbit-liver homogenates. Although no benzoic acid formation was found using rat-liver homogenates the possibility still existed that a rabbit-liver preparation might be more active, since slices of this tissue have been shown above to be more effective in aromatizing *cyclo*hexanecarboxylic acid.

The oxidation of *n*-octanoic acid by rabbit-liver homogenate was more variable, somewhat slower and more sustained than by the corresponding preparation from rat liver. *cyclo*Hexanecarboxylic acid (0·003 M) only slightly decreased homogenate respiration when used as the sole substrate, but it almost completely inhibited the ability of the homogenate to oxidize *n*-octanoic acid (Fig. 6). Similarly, Δ^1- and Δ^2-*cyclo*hexanecarboxylic acids and hexahydrohippuric acid all failed to act as oxidizable substrates—the respiration curves being almost identical with that for *cyclo*hexanecarboxylic acid shown in Fig. 6. Only traces of benzoic acid were found after the incubation of any of these substances (Table 9); these are too near the experimental limit of the estimation method to be significant. It is clear, however, that neither the rabbit-liver nor rat-liver

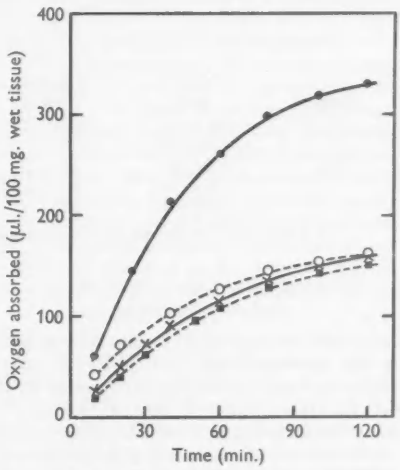

Fig. 6. Respiration of rabbit-liver homogenate in the presence of *cyclo*hexanecarboxylate. Conditions as in 'Methods'. ×—×, homogenate alone; ●—●, homogenate + 0·001 M-*n*-octanoate; ■- - -■, homogenate + 0·003 M-*cyclo*hexanecarboxylate; ○- - -○, homogenate + 0·001 M-*n*-octanoate + 0·003 M-*cyclo*hexanecarboxylate.

fortified homogenates were able to increase the aromatization reaction as they do the oxidation of straight-chain fatty acids in comparison with tissue slices (cf. Fig. 5).

Table 9. *Rabbit-liver homogenate: effect of* cyclo*hexane- and* cyclo*hexene-carboxylic acids on respiration*

Substrate	O_2 absorbed (μl.)	Benzoic acid formed during incubation (mg.)
Rabbit 1: incubation period 1 hr.		
None	75	0·00
0·001 M-*n*-octanoate	114	N.D.
0·003 M-*cyclo*hexanecarboxylate	45	0·03
0·003 M-Δ^1-*cyclo*hexenecarboxylate	67	0·01
0·003 M-Δ^2-*cyclo*hexenecarboxylate	62	0·03
0·003 M-hexahydrohippurate	60	0·01
Rabbit 2: incubation period 2 hr.		
None	115	0·00
0·001 M-*n*-octanoate	267	N.D.
0·001 M-*n*-octanoate + 0·003 M-*cyclo*hexanecarboxylate	115	0·03
0·003 M-*cyclo*hexanecarboxylate	N.D.	0·02
Rabbit 3: incubation period 2 hr.		
None	159	0·00
0·001 M-*n*-octanoate	330; 306	N.D.
0·001 M-*n*-octanoate + 0·003 M-*cyclo*hexanecarboxylate	158	0·03
0·003 M-*cyclo*hexanecarboxylate	152; 140	0·02

N.D. means not determined.

Table 10. *Urinary excretion of hippuric acid in man following oral administration of* laevo*quinic acid*

(Dose of free acid 6·0 g., given at '0 hr.'.)

Subject	Period (hr.)	Vol. urine (ml.)		Hippuric acid isolated (g.) Nearly pure	Pure	Hippuric acid isolated, increase over blank, as percentage of theoretical Nearly pure	Pure
C.T.B.	−24 to 0	2550		1·532	0·910	74	68
	0 to 24	2785		5·612	4·723		
F.D.	0 to 6	445	Total 1805	1·482	Total pure 3·330	77*	43*
	6 to 12	670		2·000			
	12 to 24	690		2·310			

* Corrections for previous 24 hr. excretion based on the above experiment have been applied.

The metabolism of quinic acid in man and other mammals

Man. Two healthy males received by mouth in water 6 g. *laevo*quinic acid of purity checked by melting point (164°) and analysis. (Found: C, 43·9; H, 6·4. Calc. for $C_7H_{12}O_6$: C, 43·8; H, 6·3 %.)

Urine was collected at intervals before and after this dose and the hippuric acid extracted from the neutralized, concentrated urine as described under Methods. After recrystallization it had m.p. 186°, unchanged on admixture with a genuine specimen. The results (Table 10) show that the increase of nearly pure hippuric acid excretion in the 24 hr. following the dose may amount to about 70 % of the theoretical amount corresponding to the quinic acid given. This excretion is shown by the second experiment of Table 10 to be spread over the 24 hr. period rather evenly, in contrast to the excretion of hippuric acid following the oral administration of benzoic acid in similar dose which, as in the familiar test for hepatic efficiency, is very much more rapid.

Species other than man. In the rabbit (Table 11) negligible residues were obtained in attempts to isolate hippuric acid from the urine after an oral dose of 2 g. quinic acid. Colorimetric estimations similarly showed only a slight increase above normal. In rats a similar result on colorimetric analysis was observed. The guinea pig after subcutaneous injection of quinic acid also showed little increase in the excretion, and the cat even showed a diminished excretion (Table 11). Consequently none of these species can be considered to aromatize quinic acid appreciably.

Tissue slices. In view of the negative results reported above it was not thought necessary to investigate slices of organs from the above species. The liver and kidney of the sheep were studied since the whole sheep has been stated to aromatize quinic acid (Vasiliu *et al.* 1940).

Slices (300 mg. fresh wt.) of liver and kidney of a recently killed sheep were incubated aerobically as usual in the presence of quinic acid concentrations from 0·04 to 0·005 M. The amounts of benzoic acid, colorimetrically estimated after incubation, were negligible (0–0·004 mg.) with both tissues.

Table 11. *Urinary excretion of hippuric (or benzoic) acid in animals following administration of* laevo*quinic acid*

Rabbits

i, By stomach tube 2 g. quinic acid to each of two rabbits. Urine passed in next 24 hr. evaporated and extracted as usual. Yield of non-crystalline residues, 22 mg., 87 mg.

ii, Another rabbit, as above, dose 1·9 g. quinic acid at 0 hr.:

Colorimetric estimations

Period (hr.)	Vol. urine (ml.)	Benzoic acid (mg.)
−24 to 0	252	15·8
0 to 24	195	45·7

Rats

(2 g. quinic acid by stomach tube distributed among 6 rats. Pooled urine collected and analysed colorimetrically.)

Period (hr.)	Vol. urine (ml.)	Benzoic acid (mg.)
−24 to 0	77	45·4
0 to 21	64	83·4
21 to 45	60	54·0

Guinea pigs

(1·874 g. quinic acid (as neutral Na salt) was injected subcutaneously, the total dose being distributed among six guinea pigs. Pooled urine analysed as above.)

Period (hr.)	Vol. urine (ml.)	Benzoic acid (mg.)
−24 to 0	316	74·4
0 to 24	415	112·0

Cats

(Two cats each received 2 g. quinic acid as a subcutaneous injection of neutral Na salt.)

	Period (hr.)	Vol. urine (ml.)	Benzoic acid (mg.)
Cat *A*	−24 to 0	355	31·9
	0 to 24	360	9·0
Cat *B*	−24 to 0	None passed	—
	0 to 24	105	8·1

Fatty acid dehydrogenase and cyclohexane carboxylic acid

A fatty acid dehydrogenase preparation was made as described by Annau (1940) and Annau, Eperjessy & Felszeghy (1942) from horse liver. From the red extract obtained after heating briefly to 60° (Annau, 1940), the fraction obtained by 0·4 saturation with ammonium sulphate was rejected and active fractions obtained by successive 0·5 saturation ('Lang enzyme', see Lang, 1939) and 0·6 saturation ('Annau enzyme'). The clear red supernatant fluid from the latter precipitate was brought to 0·7 saturation with ammonium sulphate when a further precipitate ('authors' enzyme') was obtained, of which the water-soluble part was used without dialysis in the experiment reported below.

All the above preparations, and also that of Burton (1948), were found to be somewhat unstable and generally far from satisfactory, especially as the amount of 'coenzyme' (adenylic acid, hypoxanthine, cozymase) used was in several cases found to be sufficient to act as a substrate for the reduction of a considerable part of the methylene blue employed as hydrogen acceptor. Many of these preparations contain active xanthine oxidase; a fact which does not seem to have been sufficiently emphasized in the publications cited.

Table 12. *Action of fatty acid dehydrogenase from horse liver on* cyclo*hexanecarboxylate, palmitate and laurate*

(Each Thunberg tube contained in the main part 1·5 ml. 0·3 M-potassium phosphate buffer, pH 7·45, or solution of the substrate (0·02 M) in the same buffer adjusted to same pH; 1·0 ml. 'authors' enzyme' (see text); 0·15 ml. adenylic acid solution in above buffer or 0·15 ml. buffer only. The hollow stopper contained 0·2 ml. methylene blue 0·05%; this was added after evacuation, filling with nitrogen and re-evacuation of the tubes, followed by 5 min. equilibration in the thermostat (temp. 37°). In some experiments 0·004 M-*cyclo*hexanecarboxylate was used without any alteration of the reduction time.)

Substrate	Palmitate		Laurate		*cyclo*Hexane-carboxylate		None added	
Adenosine-5-phosphoric acid (40 μg.)	–	+	–	+	–	+	–	+
Decolorization time (t, min.)	23	21	40	36	43	44·5	38	43·5
$1/t$ (mean)	0·045		0·027		0·023		0·025	

The results of action of the 0·7 saturation fraction on palmitate, laurate and *cyclo*hexanecarboxylate are shown in Table 12. With the preparation used the rate of reduction of methylene blue was increased in presence of palmitate, possibly very slightly so by laurate, and not significantly affected by *cyclo*hexanecarboxylate. Since this work was completed another fatty acid dehydrogenase preparation has also been found to be inactive towards *cyclo*hexanecarboxylate by Fantl & Lincoln (1949).

DISCUSSION

The urinary excretion of benzoic acid following the administration of *cyclo*hexanecarboxylic acid to men, dogs and rabbits was studied by Bernhard (1937) and Bernhard & Caflisch-Weill (1945). In the dog 45–59% of the dose was aromatized, in man 55–91%, and in two rabbits studied very variable results were obtained; 93·5% conversion in one animal, but only a non-crystallizable oil could be isolated from the urine of another rabbit.

The results given above (Table 1) for this reaction in the rabbit show a more consistent behaviour in our animals. The basal excretion of benzoic acid (mainly as hippuric acid) in the rabbit maintained on a diet of cabbage only is favourably low and reasonably constant. After subcutaneous injection of amounts of *cyclo*hexanecarboxylic acid within the range of 0·5–2·0 g., extra hippuric acid equivalent to 40–55% of the dose is regularly excreted in the urine (cf. Table 1). (Similar results were observed after oral administration in several unpublished experiments.) Consequently, the rabbit is in our experience a very suitable species for this investigation. The rat, on the contrary, excretes a very much lower percentage (4%) of benzoic acid following the administration of *cyclo*hexanecarboxylic acid (cf. Table 2). A marked species specificity has frequently been observed by us in these aromatization reactions, as, for example, in the behaviour of different species towards quinic acid.

In the rabbit the excretion of subcutaneously injected sodium benzoate as urinary hippuric acid is also favourable (Table 1), about 70% of a similar dose being excreted in this form in the subsequent 24 hr. This agrees well with the experience of Bray *et al.* (1946), who recovered on the average 77% of orally administered sodium benzoate as hippuric acid and 11% as glucuronide from rabbit urine. We have not distinguished between these two excretion products in our experiments, since the main object was to determine the degree of aromatization of the *cyclo*hexane derivatives.

The rapid rate of aromatization of *cyclo*hexanecarboxylic acid in the rabbit is shown not only by the fact that excretion of the benzoic acid derived from it is almost complete in 24 hr. (Table 1), but also by the rapid rise in the level of blood benzoic acid shown in Fig. 2. 2–4 hr. after the injection of an amount of

*cyclo*hexanecarboxylic acid equivalent to about 1 g. benzoic acid, the basal level is raised by a factor of 3–4·5, from which a nearly normal value is regained after about 24 hr. Whether this circulating benzoic acid is free or combined has not been investigated.

Bernhard's extensive researches on aromatization left unsolved two major points: the nature of the intermediate products by way of which the benzenoid ring arises from the fully saturated *cyclo*hexane derivatives, and the sites in the animal body at which this reaction occurs. The main object of the present paper was to investigate these two important aspects of biological aromatization.

that none of these is aromatized in the rabbit to anything approaching the extent of aromatization of *cyclo*hexanecarboxylic acid itself. The slight degree of aromatization, e.g. with *trans*-4-hydroxy*cyclo*hexanecarboxylic acid (11·7 % of dose), may perhaps be due to partial dehydration to the Δ^3 compound within the body. There is also the probability that some of these hydroxy compounds undergo oxidative degradation in the body: this is discussed in detail below under the behaviour of tissue slices and homogenates towards these compounds. In this way they may be removed from the pathways leading to aromatization.

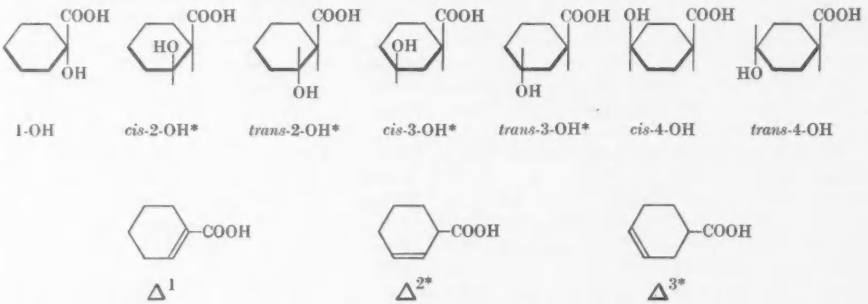

Fig. 7. Hydroxy*cyclo*hexanecarboxylic acids and *cyclo*hexenecarboxylic acids studied as possible intermediates in the aromatization of *cyclo*hexanecarboxylic acid. Compounds able to exist as optically active isomers are marked with an asterisk *.

The overall result of the aromatization of *cyclo*hexanecarboxylic acid is the formation of three unsaturated HC=CH bonds from three $—CH_2—CH_2—$ linkages. In the present work consideration has been given to two of the possible mechanisms for the stepwise formation of these bonds; one a direct process, involving dehydrogenation, the other indirect, in which the double bonds arise by the elimination of a previously introduced substituent together with a hydrogen atom. The substituent considered here is the hydroxyl group whose elimination, together with an atom of hydrogen as water, would lead to the appearance of an unsaturated link. Even simple reactions of this type offer numerous pathways to the aromatization of the *cyclo*hexane ring and Fig. 7 shows the possible compounds resulting from the introduction of either one double bond or one hydroxyl group. Subsequent steps in the aromatization might proceed by several alternative routes and a combination of two mechanisms would greatly increase the number of hypothetical intermediates. To mention only one possibility, the further dehydrogenation of the three *cyclo*hexenecarboxylic acids could lead to five possible dihydrobenzoic acids.

All seven monohydroxy*cyclo*hexanecarboxylic acids shown in Fig. 7 were injected into rabbits with results as shown in Table 1 and Fig. 1. It is evident

On the other hand, each of the three *cyclo*hexenecarboxylic acids yields much higher amounts of benzoic acid in the rabbit (Table 1 and Fig. 1), the percentage conversion of the Δ^1 and Δ^3 compounds being 60–70 %, i.e. equal to or greater than that of *cyclo*hexanecarboxylic acid itself. (The fate of these compounds in isolated tissue is described below.) Thus on the evidence derived from the intact rabbit, while the Δ^2-*cyclo*hexenecarboxylic acid appears to be aromatized too slowly to be an intermediate (cf. Table 1), either the Δ^1- or Δ^3-*cyclo*hexenecarboxylic acid could be a possible intermediate in this reaction.

It is to be noted that the hydroxy derivatives of *cyclo*hexanecarboxylic acid used were all optically inactive preparations, being the synthetic mixtures of the two forms, but even if the body were selectively to aromatize only one isomer, the resulting benzoic acid should have been easily detectable since the doses given were fairly large.

The metabolism of *laevo*quinic acid presents some points of special interest. In our experience the formation of benzoic acid from this compound is confined to man among the species studied, and it was not appreciably converted in rabbit, rat, guinea pig or cat. However, the conversion in man is remarkably high showing that at least this particular polyhydroxy derivative of *cyclo*hexanecarboxylic acid is

capable of undergoing biological reactions which must involve dehydrations and reduction to yield benzoic acid. The mechanism of this is at present quite unknown. It cannot be excluded that the intestinal flora are responsible.

Aromatization in tissue slices and homogenates

In order to obtain further evidence on the site and nature of the aromatization reaction we have investigated the behaviour of isolated tissues in the form of slices and homogenates. Bernhard & Caflish-Weill (1945, p. 1703) mention that with minced liver, kidney and muscle of dogs and horses they did not succeed in obtaining dehydrogenation of hexahydrobenzoic acid, and this is apparently the only instance of the investigation of aromatization hitherto made under these conditions. However, it was shown (Dickens, 1947) that sliced liver and kidney of the rabbit are able to effect this aromatization *in vitro*, and more detailed information is given in the present paper. Table 3 includes a number of rabbit tissues of which only the liver and to a less extent the kidney were highly active, the values for rabbit-liver slices given in Tables 4 and 5 being more typical than the unusually low figure cited in Table 3. Rabbit blood even in 10 ml. amounts gave only slight and rather dubious aromatization (see Experimental section), and here the result is perhaps confused by the considerable content of preformed colour-forming substance (*p*-aminobenzoic acid?) present in blood. Consequently the liver appears on the present evidence to be the main site of aromatization in the animal body.

The species difference noted in the whole animal is again evident in a comparison of rabbit- and rat-liver slices, the latter being less active in aromatization just as the whole rat is less efficient in this respect (cf. Tables 2, 3, 4 and 5), though the difference is less marked in the isolated tissue.

The aromatization reaction is shown by the experiments of Table 4 to be an aerobic process in rabbit-liver slices, being strongly inhibited or completely absent in non-respiring slices, whether tissue respiration is checked by anaerobic conditions or by the presence of cyanide. It is somewhat dependent on the suspension medium, being rather greater in presence of bicarbonate that in phosphate-buffered Ringer solution. As regards the effect of substrate concentration, the early results reported by Dickens (1947) have not been repeated in subsequent experiments (Table 6), although the higher concentrations of *cyclo*hexanecarboxylic acid still appears to be occasionally slightly inhibitory. The reason for these variations is not clear.

The formation of hippuric acid from *cyclo*hexanecarboxylic acid in slices of rabbit liver has been confirmed by isolation of the product, as has been described in detail in the Experimental section. There can, therefore, be no doubt that both the aromatization and conjugation with glycine do actually occur in isolated liver tissue, but whether the conjugation is subsequent to or precedes the aromatization is not known, though the former assumption seems more probable. However, hexahydrohippuric acid and hexahydrobenzamide have also been found to yield corresponding aromatic compounds in rabbit-liver slices (Dickens, 1947).

The time course of the aromatization reaction shown in Fig. 3 indicates that the reaction rate falls with time of incubation much more rapidly than the tissue respiration. Whether this is due to the accumulation of hippuric acid in the medium has not been ascertained.

We may therefore conclude that aromatization is a progressive process associated with active respiration of the tissue and, in rabbit liver at least, it is accompanied by conjugation of the benzoic acid with tissue glycine to yield hippuric acid.

Further light on the mechanism of aromatization is provided by the study of the action of tissue slices on the hydroxy*cyclo*hexane derivatives and of the three unsaturated compounds shown in Fig. 7. As in the whole rabbit, none of the seven hydroxy derivatives shown in Table 7 yields more than traces of benzoic acid when incubated with rabbit-liver slices. On the other hand, the experiments represented in Fig. 4 show that while Δ^1-*cyclo*hexenecarboxylic acid is aromatized under these conditions at only about one-third to one-half of the rate of *cyclo*hexanecarboxylic acid, the Δ^2 compound almost attains the same rate as the fully saturated compound. But by far the most vigorously aromatized of these compounds is the Δ^3-*cyclo*hexenecarboxylic acid, which in presence of respiring rabbit-liver slices yields benzoic acid at a rate much greater than *cyclo*hexanecarboxylic acid in the same liver preparation.

If the criterion be adopted that an intermediate in metabolism should be capable of transformation to the end product at a rate at least equal to that of the original substrate, Δ^3-*cyclo*hexenecarboxylic acid, alone among the compounds studied, qualifies for the role of intermediate in the aromatization of *cyclo*hexanecarboxylate. This is certainly true of the behaviour in liver slices; in the whole animal, however, either Δ^1- or Δ^3-*cyclo*hexenecarboxylic acid could be considered eligible. Possibly elsewhere than in liver the animal body can attack the Δ^1 compound, though we have no direct evidence of this. Since, however, the Δ^3 compound is the only one which both the whole animal and the liver slice are able to aromatize at a sufficient rate, it appears on the present evidence to be the most probable primary intermediate in this aromatization reaction. Of the nature of this dehydrogenation of *cyclo*hexanecarboxylic acid to the Δ^3 compound we have

as yet little information. The known fatty acid dehydrogenase preparations of animal origin do not appreciably dehydrogenate *cyclo*hexanecarboxylic acid, as we have shown, and as Fantl & Lincoln (1949) have independently found also. These dehydrogenase preparations, however, are known to be somewhat restricted in their attack to certain long-chain fatty acids.

We therefore turned to the use of tissue homogenates which are known to be capable of oxidizing a wide range of fatty acids, to see if these preparations can effect the aromatization. Rat-liver homogenates, which have been more extensively investigated hitherto than rabbit-liver preparations, gave no detectable amount of benzoic acid from any of the substrates used (Table 8) which included *cyclo*hexanecarboxylic acid, its seven monohydroxy derivatives, and all three mono-unsaturated derivatives. Hence even these preparations which are exceedingly active in oxidizing fatty acids such as octanoic acid do not have the ability to aromatize. This is not due to the use of rat- instead of rabbit-liver tissue, for the latter when homogenized similarly had almost negligible aromatizing activity (Table 9). This dependence of the aromatization reaction on the integrity of cell structure is a difficulty in the way of more detailed investigation of the enzyme mechanism concerned in aromatization.

SUMMARY

1. The conversion of *cyclo*hexanecarboxylic acid to benzoic acid, with urinary excretion of hippuric acid, has been studied in the intact rabbit. The observations of Bernhard (1937, 1938*a*, *b*) have been confirmed, but the excretion in our animals was more consistent, being equivalent to between 38–55% of the dose. In the rat it is lower.

2. The intact rabbit is unable to aromatize appreciably any of the seven possible monohydroxy derivatives of *cyclo*hexanecarboxylic acid, with the exception that *trans*-4-hydroxy*cyclo*hexanecarboxylic acid is feebly (10%) aromatized.

3. The intact rabbit aromatizes Δ^1- and Δ^3-*cyclo*hexenecarboxylic acids to an extent similar to that of *cyclo*hexanecarboxylic acid. The Δ^2-acid is somewhat less aromatized.

4. The reaction has been studied in excised tissues by means of tissue slices, homogenates and extracts.

5. Of the rabbit tissues examined in the form of slices, the liver was most active in aromatization and the kidney next in activity. The effect of varying the conditions of incubation was studied, and the product formed by rabbit-liver slices from *cyclo*hexanecarboxylic acid was proved to be hippuric acid by isolation.

6. In agreement with the behaviour of the intact rabbit, rabbit-liver slices do not form appreciable quantities of benzoic acid from any of the monohydroxy*cyclo*hexanecarboxylic acids.

7. Rabbit-liver slices are able to aromatize Δ^3-*cyclo*hexenecarboxylic acid to an extent greater than that observed with *cyclo*hexanecarboxylic acid. The Δ^1 compound was much less rapidly converted to benzoic acid, while the Δ^2 compound occupied a middle position between the other two *cyclo*hexene derivatives. Since the Δ^2 compound in the whole animal is less readily aromatized than either the Δ^1- or Δ^3-*cyclo*hexenecarboxylic acid, the latter is the only one of the three unsaturated compounds which is aromatized at a sufficient rate both in the whole animal and in liver slices for it to be a possible intermediate.

8. Homogenates of rat liver which vigorously oxidize fatty acids do not aromatize *cyclo*hexanecarboxylic acid, or any of its monohydroxy or mono-unsaturated derivatives to any considerable extent. Only the *cis*-4-hydroxy derivative was vigorously oxidized, whereas most of the other hydroxy compounds depressed the oxygen uptake of the homogenate. *cyclo*Hexanecarboxylic acid and its Δ^3 derivative (0·003M) depressed the oxidation of octanoate by liver homogenate. Rabbit-liver homogenates behaved similarly.

9. Fatty acid dehydrogenase preparations prepared from liver tissue were not able to dehydrogenate *cyclo*hexanecarboxylic acid.

10. Quinic acid (1:3:4:5-tetrahydroxy*cyclo*hexanecarboxylic acid) is converted to hippuric acid in good yield by man, but not by the other species in which it was tested.

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A Note on the Racemization of Serine

By J. C. CRAWHALL and D. F. ELLIOTT
National Institute for Medical Research, Mill Hill, London, N.W. 7

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According to Daft & Coghill (1931), the successful preparation of DL-serine from sericin by the method of Cramer (1865) depended on the intermediate mixture of serine and other amino-acids being kept for 1 hr. at room temperature in the presence of baryta at pH 9 before the final crystallization stages were carried out. These authors believed that the baryta treatment caused racemization of the serine, but no experimental evidence was produced in support of this idea. In view of the resistance to racemization by alkali observed in the case of certain amino-acids, it seemed remarkable that serine should be so labile. L-Tyrosine, for example, is completely racemized only after 30 hr. boiling with 5N-sodium hydroxide (Gunness, Dwyer & Stokes, 1946). L-Serine was required for certain investigations in this laboratory, and therefore it was a matter of practical importance as well as of theoretical interest to study the effect of alkali in more detail.

The results obtained under various conditions are shown in Table 1. It will be seen that even in the

Table 1. *Optical rotation and racemization studies of* D-*serine and* D-*alanine at various* pH *values*

Amino-acid	$[\alpha]_D^{20°}$	Solvent	c	Exp. no.	Initial $[\alpha]_D$ in NaOH† solution*	pH	Duration of racemization exp. (hr.)	Temp. (°)	Final $[\alpha]_D$ of solution*
Serine	+6·65±0·05°	Water	9·7	1	+6·4°±0·3° (18°; *c*, 1·0)	8·9	192	18	+6·4°±0·3° (18°; *c*, 1·0)
				2	+4·6°±0·3° (18°; *c*, 0·9)	9·9	18	18	+4·6°±0·3° (18°; *c*, 0·9)
							2·25	100	+4·6°±0·3° (18°; *c*, 0·9)
				3	+3·0°±0·1° (19°; *c*, 10·5)	11·4	2·5	18	+3·0°±0·1° (18°; *c*, 10·5)
							4·25	105	+2·7°±0·1° (18°; *c*, 10·5)
							17	105	+1·9°±0·1° (18°; *c*, 10·5)
				4	+3·2°±0·1° (19°; *c*, 10·0)	>13	22	18	+3·0°±0·1° (18°; *c*, 10·0)
				5†	+3·4°±0·1° (20°; *c*, 4·3)	10	18	18	+3·4°±0·1° (20°; *c*, 4·3)
Alanine	−10·0°±0·2°	N-HCl	5·8	6	−3·52°±0·1° (20°; *c*, 8·0)	12·4	16	105	−3·52°±0·1° (20°; *c*, 8·0)
	−10·7°±0·2°	N-HCl	5·8	7	−3·95°±0·1° (20°; *c*, 7·3)	13·4	16	105	−3·95°±0·1° (20°; *c*, 7·3)

* t and c values in parentheses. † Exp. no. 5, $Ba(OH)_2$ and not NaOH was used.

presence of excess of N-sodium hydroxide (Exp. 4) the optical rotation of D-serine was unchanged after 22 hr. at room temperature. The assumption of Daft & Coghill (1931) regarding the racemization of serine was therefore incorrect. On the other hand, comparison of Exp. 3 with Exps. 6 and 7 showed that D-serine was relatively easily racemized at 105° and pH 11·4, whereas D-alanine remained unchanged even at pH 13·4. It was necessary to consider the possibility that part at least of the observed fall in rotation was due to decomposition of the serine and not to racemization. Daft & Coghill (1931) have already shown that serine is slowly decomposed by hot concentrated alkali into a complex mixture of products including ammonia, pyruvic acid, alanine and glycine. In view of the vigorous conditions necessary to decompose serine it seemed probable that racemization alone had occurred in our case. This was confirmed by the observation that at pH 11·4 and 105° serine evolved no ammonia and no amino-acid except serine remained in solution.

The observation made by Daft & Coghill (1931) in their preparation of DL-serine is difficult to explain. It is possible that some racemization of the serine residues occurred during the acid hydrolysis of the protein, but in the absence of a method for the quantitative isolation of serine in an amount sufficient for accurate polarimetric measurement this must remain an open question. Serine having full optical activity can be isolated in fairly good yield from silk as the *p*-hydroxyazobenzene-*p'*-sulphonate by the method of Stein, Moore, Stamm, Chou & Bergmann (1942), but this does not shed any light on the problem because of the lower solubility of the optically active salt compared with that of the DL-isomer (see Experimental section).

EXPERIMENTAL

Resolution of DL-*serine.* *N*-*p*-Nitrobenzoylserine was prepared in 75% yield by the method used recently by Zambito, Peretz & Howe (1949) for the *p*-nitrobenzoylation of DL-threonine. This method is superior to that of Fischer & Jacobs (1906). Resolution of the nitrobenzoyl derivative was carried out according to the latter authors.

Racemization studies on D-*serine and* D-*alanine.* Optical rotations of the solutions immediately after preparation are recorded in Table 1. pH values were determined by the glass electrode. Experiments with serine at 100° were carried out in sealed tubes to avoid darkening. The serine from Exp. 3 was isolated by acidifying the solution with conc. HCl, evaporating to dryness under reduced pressure, extracting the residue with hot ethanol, filtering and adding conc. ammonia solution to the filtrate. After cooling, the serine was collected and crystallized from aqueous ethanol. The yield was 0·21 g. of serine, $[\alpha]_D^{20°} + 3·9° \pm 0·4°$ in water (*c*, 1·5); the experiment was commenced with 0·6 g. serine. This sample was therefore 42% racemized, comparing well with the value of 35% expected from the rotation of the solution.

The stability of DL-serine under the conditions of Exp. 3 was demonstrated as follows. A pure sample of DL-serine (0·6 g.) and N-NaOH (5·7 ml.) were placed in one limb of a double-limbed tube. The second limb contained 2 ml. of N-H_2SO_4 which was approximately isopiestic with the serine solution. After 17 hr. at 105°, back-titration of the H_2SO_4 revealed that not more than 1% of the serine nitrogen had been evolved as volatile material. The alkaline serine solution was neutralized with HCl and submitted to paper chromatography using aqueous phenol as solvent; as a control, a solution of serine containing the same amount of NaCl as the unknown was used. No other amino-acid was found in the alkali-treated serine, and the two serine spots were in identical positions on the chromatograms.

Determination of the solubilities of p-*hydroxyazobenzene*-p'-*sulphonic acid salts of* DL- *and* D-*serine at* 20°. The pure salts were dissolved in hot water and the solutions allowed to crystallize for 16 hr. Measured volumes of the supernatant were evaporated to dryness in a vacuum desiccator and the residue weighed. Experiments were carried out in duplicate. Solubility was also determined by shaking the powdered solids for 16 hr. with water, then filtering and proceeding as before. The solubility of the DL-salt was found to be 3·5 g. ±0·1 g./100 ml. of solution; that of the D-isomer was 1·9 g. ±0·1 g./100 ml. of solution.

SUMMARY

Serine was not racemized at pH 9 at room temperature as Daft & Coghill (1931) supposed, neither was it racemized by excess of N-NaOH at room temperature. On the other hand, it was found to be racemized at 105° and pH 11·4, whereas alanine was unchanged at pH 13·4 and 105°.

The authors wish to thank Mr A. D. Brownstone for experimental assistance.

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Ultramicro Determination of Chloride

By R. VISWANATHAN
Central Marine Fisheries Research Station, Mandapam Camp, South India

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A simple technique has been described by Wigglesworth (1937) for the determination of chloride in as little as 0·3 μl. of fluid. This is based on the Volhard titration and has been used in this Laboratory since 1947 for the examination of fish and crustacean blood. While the results obtained were satisfactory, the tedious back titration and the comparatively impermanent end point were found to be inconvenient when a large number of biological samples had to be dealt with. The necessity was therefore felt for an alternative procedure which would be free from these limitations.

The method here described is an adaptation of the procedure outlined by Van Slyke & Hiller (1947) suitably modified for use with drop-scale volumes.

METHODS

Apparatus

Waxed capillary pipettes and burettes of the type described by Wigglesworth.

Constriction 'Carlsberg' pipettes (Glick, 1935) with paraffined tips for delivering tungstate-phosphoric acid reagent, KI and starch solutions and of capacity 25, 25 and 15 μl. respectively.

Reaction tubes. Glass tubes (length 20 mm., internal diameter 3–4 mm.) were coated internally with paraffin. Molten paraffin was allowed to solidify near the middle of the tube so that two cavities were formed, one of slightly larger capacity than the other. For stoppering the tube plasticine was used. After use, the paraffin could be pushed out of the tube and the latter rewaxed after suitable cleaning.

Waxed glass plates.

Reagents

Sodium tungstate 0·6% (*w/v*) *in* 0·15 M-*phosphoric acid* (Van Slyke & Hiller, 1947).

Silver iodate (Sendroy, 1937). It is essential that this be kept in a brown-glass bottle and preserved in a desiccator.

Potassium iodide. 20 g. KI in 100 ml. aqueous solution (containing a drop of dil. NaOH). The solution is kept in a brown-glass bottle.

Starch. 1% (w/v) solution of soluble starch in 20% (w/v) NaCl (prepared fresh every week).

Sodium thiosulphate. Stock 0·1 N-$Na_2S_2O_3$ solution containing 1 g. borax/l. of solution.

Standard sodium chloride solutions. Prepared by dissolving NaCl (Analar), previously dried at 120° for 12 hr., in distilled water.

Procedure

Standard sodium chloride solutions. 5 'units' of the chloride solution (about 1 μl.) were delivered from a graduated capillary pipette into the bigger chamber of the reaction tube, containing 25 μl. of tungstate-phosphoric acid reagent. 1 μg. of $AgIO_3$ was next tipped into the reaction chamber from the end of a horn spatula. This amount of iodate was enough to form a small layer at the bottom of the reaction chamber and could be fixed by practice. The reactants were stirred for 40 sec. with the sealed and paraffined end of a drawn-out glass tube, and the tube was stoppered with plasticine. The contents of the reaction tube were allowed to settle for 30 min. Suitable samples were then pipetted on to the surface of a waxed glass plate, and 25 μl. of 20% KI and 15 μl. of the starch indicator solution were placed alongside the sample drop on the glass plate. The KI and starch were mixed by an air jet and the drop mixture was directed on to the sample. The final drop mixture was titrated with 0·01 N-$Na_2S_2O_3$ solution (prepared every 2 days by tenfold dilution of the stock solution) from a capillary burette. A water blank was run using 5 units of distilled water instead of the chloride solution, and this blank value was subtracted from the titres of the chloride solution.

The standard solution contained 1·4% (w/v) NaCl (239·3 m-equiv./l.) for the range 0·7–2·8% NaCl (119·8 m-equiv./l. to 478·6 m-equiv./l.) and 0·35% (w/v) NaCl (59·9 m-equiv./l.) for NaCl concentrations below 0·7%. The size of the sample used in the former range was such that the standardization titre was about 35 units of 0·01 N-$Na_2S_2O_3$ solution. For the latter range the sample was thrice this size.

It was found convenient to have six reaction mixtures set up while six titrations were being carried out. In this manner twelve titrations could be completed in 2·5 hr.

Chloride in blood serum. The procedure was similar to that described for standard NaCl solutions, but the estimations were carried out (in duplicate or in triplicate) at the 60–120 mM-level, and where necessary the serum was diluted. Two samples of human blood serum analysed 98·31 and 97·59 mM-chloride respectively. The corresponding figures by the Wigglesworth method were 98·86 and 101·7 mM-chloride.

Recovery of NaCl added to serum varied from 95 to 103% in the range above 120 mM; and from 99 to 107% in the range 18–60 mM (Table 3).

RESULTS

From Table 1 it is seen that the error does not exceed 3% in the range 120–600 mM; while below 50 mM the accuracy is within 5% of the correct value.

In Table 2 are given the results obtained by periodical checks on the Wigglesworth method.

DISCUSSION

In Sendroy's (1937) micro method, it is necessary to correct for the solubility of silver iodate in varying chloride concentrations. In the drop-scale modification outlined above, this correction is substituted by the usual type of 'water blank' titre for the added

Table 1. *Drop-scale iodometry with standard sodium chloride solutions*

NaCl (m-equiv./l.)			
	Found		
Calc.	Mean	S.D.	No. of determinations
598·8	600·6	9·56	6
479·2	483·0	2·31	6
359·4	352·6	7·63	6
119·8	116·3	3·66	6
47·92	45·83	2·29	6
35·94	34·95	2·76	6
23·96	23·66	5·33	6
11·98	11·93	0·53	6

Table 2. *Drop-scale Volhard estimations with standard sodium chloride solutions*

NaCl (m-equiv./l.)			
	Found		
Calc.	Mean	S.D.	No. of determinations
513·2	501·7	4·87	10
342·7	340·7	8·88	14
279·7	276·4	2·86	12
273·7	285·9	4·32	10
171·1	168·3	6·75	15
139·9	137·6	3·91	12
136·9	143·1	2·84	10
85·55	83·71	8·64	16
69·95	65·21	7·39	16

silver iodate. Further, the minimal (final) concentrations of chloride which react with silver iodate are different in the two methods. In Sendroy's, it is 0·01 M (according to Judah, 1949); and in the present method, 0·0005 M (Table 1).

From a comparison of the figures in Tables 1 and 2 and from the serum chloride values by the two methods, it is seen that the present method and that of Wigglesworth are about equally accurate. Nevertheless, the iodometric procedure would appear to be more convenient for at least two reasons. It is less time consuming, and there is a sharp end point over a wider range of chloride concentrations.

Table 3. *Recovery of sodium chloride added to serum*

(Drop-scale iodometry.)

NaCl (m-equiv./l.)		NaCl (m-equiv./l.)	
Added	Recovered	Added	Recovered
299·4	285·2	59·88	60·54
239·6	228·3	29·94	31·96
179·7	175·3	23·96	24·88
119·8	122·7	17·97	17·82

SUMMARY

An adaptation of the Van Slyke and Hiller method has been described for the ultramicro determination of chloride.

My thanks are due to Dr N. K. Panikkar for his interest in this work and to the Chief Research Officer, Central Marine Fisheries Research Station, Mandapam Camp, for permission to publish this article.

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Improved Manometric Fluid

By H. A. KREBS
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield

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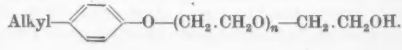
The manometric fluid in common use in Warburg manometers (Brodie, 1910; Warburg, 1912) contains bile salts to faci itate the free flow of the fluid in the capillary. Many of the wetting agents which have become available in recent years are much more effective than bile salts, and it is therefore possible to design an improved manometric fluid. In testing a series of detergents it was found that some compounds, e.g. alkyl sulphates and cetyltrimethylammonium bromide, are liable to form a stable froth, and for this reason are not suitable for use in manometer fluids. Suitable substances were found among the non-ionic detergents, in particular among those arising by condensation of alkyl phenols with ethylene oxide, of the following general structure



Lissapol N (Imperial Chemical Industries Ltd., Blackley, Manchester), Stergene (Domestos Ltd., Newcastle-upon-Tyne) and Triton X-100 or Triton A-20 (Rohm & Haas Co., Philadelphia, Pa.) belong to this group of 'alkylarylpolyethoxyethanols'.

The following fluid, which has a specific gravity of 1·033 at 20°, is recommended.

Sodium bromide (anhydrous)	44 g.
Stergene (or Lissapol N)	1 g.
Evans blue	0·3 g.
Water	to 1000 ml.

If filtration is required, sintered-glass funnels should be used as filter paper removes some of the dye by adsorption.

Sodium bromide is used in preference to sodium chloride or potassium chloride because the detergent solution gradually develops a turbidity (not visible in the presence of the dye) on addition of the chlorides, and precipitates are formed on storage in mixtures of detergent, alkali chloride (especially potassium chloride) and Evans blue. Sodium bromide is free from this disadvantage. Commercial sodium bromide usually contains moisture and should be dried at 105–110° before weighing. The figure given for Stergene and Lissapol N refers to the commercially available syrupy liquid which contains about 70 % water. As Triton X-100 and Triton A-20 are practically water-free, 0·3 g. of either of these detergents is equivalent to 1 g. of the other two. Evans blue, widely used for blood volume determinations (Dawson, Evans & Whipple, 1920), and first recommended for manometer fluids by Bain (1945), has proved more stable than other dyes tested. It is not taken up by the rubber or plastic reservoirs.

The new fluid has a much greater cleansing power than Brodie's solution. It keeps better than the older fluid which sometimes becomes viscous on storage owing to changes in the bile salts. Froth and bubbles formed in the manometer capillary disappear more readily than from Brodie's solution. The greater cleansing power was assessed by placing in the manometer fluid a grease-coated microscopic slide prepared by dipping it into ether containing 1 % lanolin.

SUMMARY

An improved manometer fluid is described in which the bile salts recommended by Brodie are replaced by a non-ionic detergent of the 'alkylarylpolyethoxyethanol' type.

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The Lipid Mobilized by Anterior Pituitary Extract

By J. CAMPBELL and C. C. LUCAS

Banting and Best Department of Medical Research, University of Toronto

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When certain anterior pituitary extracts are administered to fasting rats a rapid increase in the lipid of the liver and kidneys, and a decrease in that of the other tissues, occurs (Best & Campbell, 1936, 1938). Since more lipid disappears from the depots than accumulates in liver and kidney, it is apparent that increased utilization of lipid accompanies the mobilization. In this study the sources and nature of the lipid mobilized were determined in mice, and certain observations on the distribution of lipid in the body were made. A similar, but less complete, study was made of the effects of the extract when given to fasting rats.

METHODS

Fifty normal adult male mice (raised on a diet of 'Purina' fox chow, supplemented with brown bread and milk) were separated into five groups. The mice were fasted from the beginning of the experiment, but water was always available. Four hours after the beginning of fasting the animals of group 1 (controls) were sacrificed. At this time each animal in groups 2 and 4 (injected controls) was given subcutaneously 9 mg. of an inert protein, bovine plasma albumin (BPA); and each animal of groups 3 and 5 was given 9 mg. anterior pituitary preparation (APP). The mice of groups 2 (BPA) and 3 (APP) were killed 7 hr. after these injections, i.e. 11 hr. after the beginning of the experiment. Mice of groups 4

and 5 were given second injections of BPA and APP, respectively, at this time, and were sacrificed 13 hr. later, i.e. 24 hr. from the beginning of the experiment. The lighter animals were chosen for group 1, since they were to be subjected to the shorter fast. Those in the other groups weighed 20–30 g. The APP was prepared from ox pituitary glands as described by Best & Campbell (1936) and the BPA was obtained from the Armour Laboratories, Chicago. These preparations were dissolved in saline at pH 7·5, and 0·5 ml. solution were injected.

RESULTS

Effect of APP on the total lipid of all tissues

The total lipid of the entire body was decreased by the administration of APP (Table 1). This indicates that, in mice, increased utilization of lipid occurred under the stimulus of the APP, as had been found previously in rats (Best & Campbell, 1938). The

Table 1. *Effect of anterior pituitary preparation on the body lipid of mice*

(Mice, ten per group, were fasted for 4 hr. before the first injection, and thereafter. The lipid composition of the mice is given per 100 g. fat-free solids of the entire bodies.)

Group no.	Injection	Fast after first injection (hr.)	Actual weights of: Entire body (g.)	Actual weights of: Fat-free solids (g.)	(g./100 g. fat-free solids of entire body): Lipid	Phospholipin	Free cholesterol	Cholesteryl ester	Glyceride
1	None	0	21·4	5·04	37·1	7·10	0·99	0·54	28·5
2	BPA	7	22·5	5·37	38·2	6·45	1·02	0·48	30·1
3	APP	7	25·0	6·07	31·6	5·80	1·00	0·50	24·4
4	BPA	20	22·4	5·59	31·2	6·09	0·98	0·68	23·1
5	APP	20	22·6	5·55	27·9	5·91	1·01	0·48	20·5

The lipid was extracted and analysed according to Best, Lucas, Patterson & Ridout (1946).* The animals were weighed, killed by crushing the occiput and the tissues were combined for analysis as follows: (1) The spleen and gastro-intestinal tract. (2) The genital organs, including the testes, epididymis, seminal vesicles and prostate gland. (3) The skin, except for the small portions adherent to the paws and tail. (4) All the adipose tissues visible to the naked eye, as detailed by Reed, Yamaguchi, Anderson & Mendel (1930), including the mesenteric, perirenal, perigenital and other adipose tissues in and about the abdominal cavity, the subcutaneous and interscapular fat tissue and the intermuscular deposits in the pelvic and scapular regions. Reed, Anderson & Mendel (1932) found that, in rats, lipid changes in these tissues tended to be similar. (5) The brain (not including the optic and olfactory nerves or the spinal cord). (6) The trachea, lungs and thymus. (7) The carcass, which consisted of the tissues remaining after those mentioned above and the liver, kidneys and adrenals had been removed. The carcass thus consisted chiefly of muscle and bone plus the heart and peripheral nerves. All these tissues, which are referred to as the 'entire body' constituted about 94% of the live weight; while lost bile, blood and urine accounted for the remaining 6%.

The methods used in the experiment on rats were essentially similar, the differences being described in the text.

* On cooling the clear light petroleum solutions of the initially soluble lipid from intestines and spleen and from brain, an amorphous, white material settled out and tended to redissolve on warming slightly. This small precipitate, when present, was removed by centrifuging. It was subsequently found to be rich in phosphorus and exhibited certain characteristics of sphingomyelin or hydrolecithin (cf. Hunter, 1942). The values presented for the phospholipin of these tissues are consequently, in some cases, low; but the amount lost was a small proportion of the total and does not invalidate the general conclusions drawn.

reduction was most pronounced in the glyceride fraction. The cholesterol and cholesteryl esters of the entire bodies were apparently not influenced significantly by the administration of the APP. Phospholipin appeared to be decreased somewhat, although the differences are of questionable significance. Expressed as percentage of the fresh weights of the entire bodies, the total lipid in groups 1–5, respectively, were 8·74, 9·10, 7·68, 7·78 and 6·85; the fat-free solids similarly expressed were 23·5, 23·9, 24·3, 25·0 and 24·5; and the water contents were 67·8, 67·0, 68·0, 67·2 and 68·8.

Mobilization of lipid

The administration of the APP produced an increase in the lipid of the liver, but only a slight indication of increase in kidney lipid. The lipids of adipose tissue, carcass and skin, in this order, were most heavily drawn upon in the mobilization produced by the APP (Fig. 1). These lipids were decreased by fasting alone, but the administration of the APP accelerated the loss. The genital organs, intestine and spleen, and the lungs and thymus contributed relatively little to the amount of lipid mobilized. The lipid of the brain was apparently not affected.

It appears that these changes in total lipid were due chiefly to changes in glycerides, which were withdrawn from adipose tissue, carcass and skin, and which were deposited in the liver (cf. Figs. 1 and 2). Of the glyceride lost by the tissues mentioned, about one-fifth could be accounted for by that which accumulated in the liver. Presumably about four-fifths had been oxidized.

As a result of the influx of glyceride into the liver following the injection of APP, the phospholipin (expressed as percentage of the fresh weight) was decreased. Further studies are required before conclusions can be drawn regarding changes in the absolute amounts of phospholipin or of cholesterol in the tissues under these conditions.

the skin contained about one-half, and the liver the next largest amount (5–6 %). The remaining 11 % was distributed among the other tissues.

Somewhat more than one-half of the phospholipin of the body occurred in the carcass; the liver contained the next largest portion (15 %), the brain contained about 6–7 % and the skin a similar

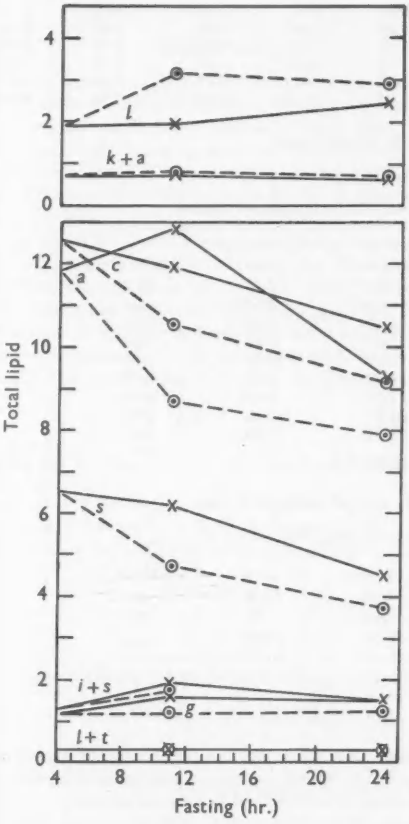

Fig. 1.

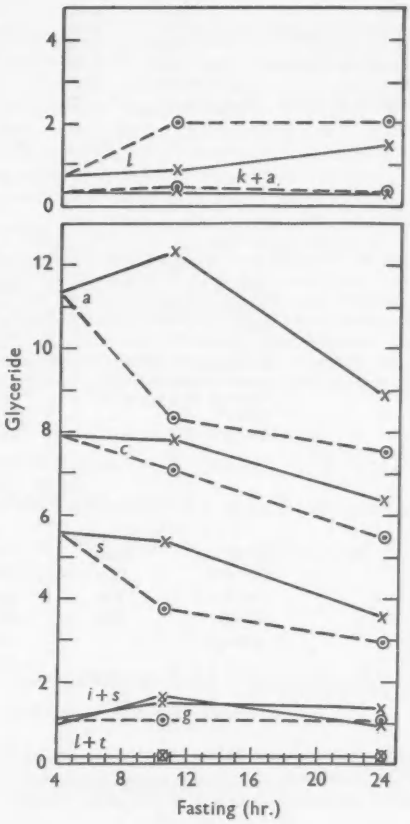

Fig. 2.

Figs. 1 and 2. The total lipids and glycerides of mouse tissues, g./100 g. fat-free residues of the entire animal. The fasting mice were injected with APP, ⊙ - - - - ⊙; or BPA, × —— ×. The tissues indicated are *l*, liver; *k+a*, kidney and adrenal; *a*, adipose tissues; *c*, carcass; *s*, skin; *g*, genital organs; *i+s*, intestine and spleen; *l+t*, lungs and thymus.

Distribution of lipid

The distribution of lipid in the bodies of mice of group 1 is shown in Table 2, the values for the other groups being similar. The carcass and the adipose tissues each contained about one-third of the total lipid of the entire body. Of the remaining one-third

amount. The largest proportion of the free cholesterol (41 %) occurred in the carcass; the skin, although second in this respect, contained less than one-half as much. The skin contained about 60 % of the cholesteryl esters and the carcass about 25 %. The adipose tissues contained about 40 % of the glycerides of the entire body, the carcass about 28 %

Table 2. *The distribution of lipid in mice*

(Group 1, fasted 4 hr. The values are given as percentages of the total amounts of each of the components in the entire animal, i.e. each vertical column totals 100%.)

Tissue	Wet weight	Fat-free solids	Water	Total lipid	Phospho-lipin	Free cholesterol	Cholesteryl ester	Glyceride
Carcass	54·3	60·9	54·7	33·9	57·1	41·1	26·5	28·0
Adipose tissues	6·59	3·10	4·48	31·8	5·40	5·16	0·55	40·0
Skin	13·5	17·2	11·7	17·7	6·30	16·6	59·8	19·8
Liver	6·50	6·15	6·70	5·20	15·1	6·80	3·11	2·60
Intestine and spleen	12·25	8·20	14·7	3·53	2·40	11·1	4·80	3·50
Genital organs	1·97	1·18	2·09	3·25	1·87	1·74	1·47	3·61
Kidneys and adrenals	1·84	1·28	2·02	1·77	3·97	4·9	1·10	1·20
Brain	1·68	0·87	1·93	1·92	6·11	10·7	1·94	0·70
Lungs and thymus	1·42	1·03	1·62	0·87	1·76	2·0	0·74	0·60

Table 3. *Composition of the tissue lipids*

(Group 1, mice fasted 4 hr.)

	Percentage of total lipids of tissue			
Tissue	Phospholipin	Free cholesterol	Cholesteryl ester	Glyceride
Carcass	32·4	3·27	1·13	63·6
Adipose tissue	3·28	0·434	0·025	96·6
Skin	6·90	2·55	4·90	85·8
Liver	57·1	3·57	0·88	38·4
Intestine and spleen	13·4	8·60	2·00	76·5
Genital organs	11·1	1·43	0·72	86·7
Kidneys and adrenals	42·4	4·27	0·76	52·5
Brain*	54·3	14·9	1·42	29·3
Lungs and thymus	38·8	6·17	1·35	53·7

* The averages of groups 2–5.

Table 4. *Rats, female, fasted and injected daily for 3 days*

Substance injected	Body weight, initial (g.)	Fatty acids (mg./100 g. body wt.)			
		Carcass	Intestine	Liver	Kidney
APP	176	4460	330	504	41
Saline	185	5250	441	131	35
Change		−790	−111	+373	+6
		−901		+379	
Unaccounted for		−522			

and the skin about 20%. Greater amounts of lipid, free cholesterol, phospholipin and water were found in the carcass than in any of the other tissues.

Composition of the lipid

Of the lipid of the carcass, somewhat less than two-thirds was present as glyceride and one-third as phospholipin; while the free and bound cholesterol together amounted to about 4% (Table 3). The lipid of skin was rich in glycerides (86%), but its outstanding characteristic was a high concentration of cholesteryl ester, which exceeded the concentration of free cholesterol. The lipids extracted from liver and from kidney and adrenals were conspicuously rich in phospholipin.

It would appear that the rate of mobilization of lipid may be related to the glyceride content of the tissue. For example, the percentage of glyceride in the lipids of adipose tissue, genital organs, skin and carcass were 97, 87, 86 and 64 respectively, while the losses of lipid from each of these tissues under the influence of the APP during 11 hr. fast, when expressed as the percentage of lipid in the fasted controls, were 32, 26, 24 and 12, respectively. The corresponding losses of glycerides, expressed as percentages of the glycerides in the controls, were 32, 28, 30 and 9, respectively.

Fatty acid mobilization and utilization in rats

The following experiment demonstrates (in confirmation of Best & Campbell, 1936), that the injection of APP produces increased mobilization and utilization of lipid in fasting rats. Adult rats, 10 per group, were fasted and injected daily for 3 days with

APP, while the control rats were given equivalent amounts of saline. The total crude fatty acids (fatty acids plus unsaponifiable lipid) were then determined (Table 4). In the group given APP, 379 mg. crude fatty acids per 100 g. initial body weight were gained by liver and kidney, while 901 mg. were lost by the other tissues. The difference of 522 mg. had presumably been utilized.

DISCUSSION

It was demonstrated by Barrett, Best & Ridout (1938) that the administration of APP to fasting mice caused rapid mobilization of lipid from the depots with an accumulation of lipid in the liver. Stetten & Salcedo (1944) demonstrated this effect in rats. The results of the present study in mice and rats support and extend these findings.

The glyceride fraction was principally affected in the mobilization of lipid produced by the anterior pituitary stimulation. Glycerides were removed from the depots, were deposited in liver, and were utilized to a greater extent than any other lipid fraction examined. While no attempt is made to explain the mechanism of lipid mobilization, it seems probable from the evidence available that the rate of mobilization from a tissue may be related to the glyceride content of that tissue.

The liver and, to a much less extent, the kidney were the only structures of those examined whose lipid contents increased during the period of lipid mobilization.

SUMMARY

The mobilization, accumulation in the liver and utilization of lipid in fasting mice injected with an anterior pituitary preparation involves chiefly the glycerides. The amount of lipid transferred from a tissue under these conditions appears to bear a relation to the glyceride content of the lipid of the tissue. The rat appears to respond similarly to treatment with anterior pituitary preparation.

We wish to express our appreciation and thanks to Prof. C. H. Best for his interest and encouragement. We are glad to acknowledge the aid of Dr M. F. Clarkson, who took an active part in the investigation, and of our colleagues Dr Jessie H. Ridout and Dr Jean M. Patterson, who assisted particularly with the phosphorus and cholesterol determinations. Part of the expenses of the investigation have been defrayed by funds supplied by the Banting Research Foundation.

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The Effect of a High-Sucrose Diet on the Calcium and Phosphorus Content of the Enamel and Dentine of Rat Incisors

By R. L. HARTLES
Biochemistry Department, School of Dental Surgery, University of Liverpool

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So far as is known no investigation has been carried out to determine the effect of a large excess of a major dietary constituent on the process of mineralization. The work reported here was concerned with investigating the effect of a diet containing 67% sucrose on the calcium and phosphorus content and the calcium/phosphorus ratio of rat incisors. In recent years such a diet has been used for the experimental production of dental caries in the white rat, the hamster and the cotton rat (Sognnaes, 1948*a*, *b*; Shaw, Schweigert, Elvehjem & Phillips, 1944). It is of interest, therefore, to know if teeth produced on a high-sugar diet differ chemically from those of animals receiving a typical stock laboratory ration, when both diets contain adequate amounts of the known vitamins and necessary minerals. Work in the past appears to have been concerned, in the main, with deficiencies of vitamins and minerals, several factors having been shown to influence the structure and composition of growing teeth. In general, however, dietary deficiencies need to be much more severe to influence the composition of the teeth than similarly to affect bone. For example, diets which cause severe rickets may produce only slight changes

in the teeth (Templin & Steenbock, 1933; Gaunt & Irving, 1939, 1940; Smith & Light, 1945).

Gies & Perlzweig (1916) and Toverud (1923) showed that, in the rat, diets low in calcium produced weak incisors having a reduced mineral content. Prolonged deficiency of vitamin A results in severe changes in the rat incisor (Smith & Lantz, 1933); lack of vitamin D in the same species gives rise to a decreased total ash and calcium content (Karshan & Rosebury, 1933), and the incisor becomes distorted (Boyle & Wesson, 1943). Recently, Sobel & Hanok (1948) have shown in the white rat that variations of the calcium and phosphorus contents of serum lead to small variations in the calcium and phosphorus of the upper incisors.

The incisor teeth of the rat (unlike the molars) grow continuously throughout life and can therefore be used to study the influence of diet upon mineralization. No evidence can be found to suggest that the actual processes of mineralization in incisor teeth differ fundamentally from those in molar teeth. It appears reasonable to assume, therefore, that conditions which affect the incisors would also affect the growing molars during their period of development.

The results reported here show statistically significant differences in the calcium and phosphorus content of the incisors of two groups of animals given a stock diet and a high-sugar diet. A parallel study is being carried out on bone and will be reported later.

EXPERIMENTAL

Animals. Albino rats from our own inbred colony were used. All animals were born to parents receiving a stock diet of Lever rat cubes.

Group 1. Twenty-two animals were weaned at 21 days, maintained on the rat cube diet and killed when 91–95 days old.

Group 2. Twenty-eight animals were weaned at 21 days on to the diet containing 67% sucrose and killed when 91–95 days old.

Diets. The stock diet of Lever cubes is, in the experience of this and other laboratories, nutritionally sound; rats grow and breed well when it is the sole source of food. The diet is made up as follows:

	(%)
Wheat germ	2·8
Skim milk powder	3·27
Dried yeast	3·74
Fine bran	29·26
Broad bran	7·47
Molasses	11·21
Coconut cake meal	16·81
Groundnut cake meal	5·60
Maize	10·28
Fish meal	5·60
Dried blood meal	1·40
Limestone	0·93
Common salt	0·70
Bone flour	0·93

The diet contains 1·41% Ca and 0·80% P. The high-sucrose diet was essentially the same as that used by Sognnaes (1948*a*) and Shaw *et al.* (1944), except that groundnut oil was used in place of corn oil, and 'Hepamino' (Evans Medical Supplies), a whole-liver preparation, replaced the liver concentrate. Each kg. of diet contained:

	(g.)
Sucrose (granulated)	670
Casein (Glaxo; unextracted)	200
'Hepamino'	40
Groundnut oil	50
Salt mixture (Osborne & Mendel, omitting NaF)	40

The following supplements were added to each kg. of diet:

	(mg.)
Aneurin	5
Riboflavin	5
Pyridoxine	5
Nicotinamide	25
Calcium pantothenate	25
p-Aminobenzoic acid	500
Choline chloride	1000
Calciferol	40 μg.
Carotene	20 mg.
α-Tocopheryl acetate	20
2-Methyl-1:4-naphthoquinone	20

The diet contains 0·87% Ca and 0·50% P.

The diets and water were fed to the animals *ad lib.* The experimental period (10 weeks) was long enough to allow for the complete replacement of the continuously growing incisors; any differences should therefore be noticeable at the end of this period. The animals were weighed twice a week and at the end of the experiment were killed with ether, the heads removed and placed in 96% ethanol.

Treatment of teeth. The upper and lower incisors of each animal were removed from the jaws, stripped of adhering tissues, split into fragments and placed in absolute ethanol overnight. The teeth were then extracted with ether in a Soxhlet apparatus for 6 hr. and dried at 110° for 3 hr. The dry fat-free incisors of each animal were pulverized in a percussion mortar so as to pass a 60-mesh sieve. The powdered teeth were fractionated into their several components by the flotation method of Manley & Hodge (1939). First, pulpal material, tissue debris and cementum were floated off in a bromoform-acetone mixture (sp.gr. 2·06) and the remaining enamel and dentine separated by flotation in a mixture of sp.gr. 2·60. Manley & Hodge (1939) recommend the use of a liquid of sp.gr. 2·70 for separating human enamel and dentine, but the corresponding rat tissues appear to be slightly less dense and in a liquid of sp.gr. 2·70 practically no enamel separates. At sp.gr. 2·65 small particles of pigmented enamel were retained in the dentine fraction; at sp.gr. 2·60 they were absent.

Use of fluorescence excited by ultraviolet light as an indication of the purity of the enamel and dentine samples. When teeth are irradiated with ultraviolet light they fluoresce. The dentine fluoresces intense blue-violet, the enamel a much paler blue and the pigmented labial surface of the enamel not at all. On examining the two fractions any brightly fluorescing granules in the enamel sample indicate the presence of dentine; conversely any dull granules in the dentine show the presence of enamel. When flotation is carried out at sp.gr. 2·60 the separation of the two tissues appears to be complete.

Analytical procedure. Samples of the enamel (10–20 mg.) were dissolved in 5 ml. of 5 N-HCl, the solution made alkaline with NH_3, re-acidified with acetic acid and made up to 25 ml. The dentine samples (50–100 mg.) were dissolved in 10 ml. 5 N-HCl, treated as for the enamel and made up to 50 ml. There is a residue after treatment with HCl, since enamel contains about 1% and dentine some 20% protein. Any Ca or P which is bound to this protein is not analysed. The analytical figures refer to the acid-soluble Ca and P in enamel and dentine.

Calcium determinations. Suitable samples of the filtered enamel and dentine solutions were analysed by precipitation of the Ca as oxalate in 15 ml. centrifuge tubes. Further operations of centrifuging, washing and titration with 0·01 N-$KMnO_4$ were all carried out in the centrifuge tube.

Phosphorus determinations. Suitably diluted portions of the filtered enamel and dentine solutions were analysed for phosphorus by the method of Berenblum & Chain (1938) using a Spekker photoelectric absorptiometer.

Both methods gave reproducible results with an accuracy within 2%.

RESULTS

The average growth rates for the two groups of animals were almost identical.

		Females (g./day)	Males (g./day)
Group 1	Stock diet	1·70	2·27
Group 2	Sugar diet	1·75	2·31

The general health of both groups was good, and the diets were readily eaten.

The results for the calcium and phosphorus analyses and the calcium/phosphorus ratios are given in Table 1. The ratios are expressed stoicheiometrically.

The percentage of calcium and phosphorus in the dry fat-free enamel and dentine of rat incisors was greater when the animals were maintained on the high-sugar diet. The ratio of calcium/phosphorus was, however, lowered. All these differences are statistically significant, the probability of their being due to chance is in all cases less than one in twenty and in four of the six cases less than one in a hundred.

DISCUSSION

The ingestion of large amounts of refined carbohydrates and especially of fermentable sugars is considered to be an important factor in the aetiology of dental caries (Jay, 1940). There are three possible ways in which a high-sugar diet may influence this condition. First, it has been shown that an increased intake of sugar increases the acid-producing oral flora (Jay, 1940; Becks, Jensen & Milarr, 1944). Secondly, the composition of the salivary secretions may be changed, although no work seems to have been done on this aspect of the problem. Thirdly, the tooth itself may undergo changes in composition if a high-sugar diet is provided during the period of tooth formation. Recently, Sognnaes (1948*a*, *b*) has reported that rats and hamsters show increased susceptibility to dental caries when maintained on a diet containing 67% sucrose. They grew and reproduced well on the diet, indicating that it contained adequate amounts of fats, proteins, vitamins, etc. He made the important observation that this susceptibility was enhanced if the high-sugar diet was fed during gestation and lactation, so that the animals were exposed to the effects of the diet during the whole of their pre- and post-natal life. When the diet was continued through three generations there was a further progressive increase in caries susceptibility. It appears, therefore, that a high intake of sucrose exerts an influence other than by its affecting the oral flora; Sognnaes (1948*a*) is of the opinion that the effect may be a systemic one.

The results of our experiments show that the enamel and dentine of the incisor teeth of animals maintained on a purified diet containing 67% sucrose had significantly higher amounts of calcium and phosphorus than the same tissues of animals

Table 1. *The calcium and phosphorus content as percentage of fat-free dry weight of rat incisor teeth on a stock diet and on a high* (67%) *sucrose diet**

	Ca (%) Mean ± S.E.M.	P (%) Mean ± S.E.M.	Ca/P stoicheiometric ratio Mean ± S.E.M.	Ca (%)/P (%) (approx. mean)
Enamel, stock diet	33·17 ± 0·37	15·99 ± 0·15	1·607 ± 0·014	2·07
	(22)	(22)	(22)	—
	29·25–35·86	14·42–17·30	1·491–1·740	1·92–2·24
Enamel, sugar diet	35·06 ± 0·31	17·27 ± 0·11	1·569 ± 0·012	2·02
	(28)	(28)	(28)	—
	31·53–37·43	15·49–18·12	1·411–1·678	1·82–2·17
Dentine, stock diet	26·00 ± 0·29	13·54 ± 0·16	1·486 ± 0·012	1·92
	(22)	(22)	(22)	—
	23·09–28·95	11·96–14·64	1·352–1·590	1·74–2·05
Dentine, sugar diet	26·75 ± 0·23	14·57 ± 0·12	1·425 ± 0·014	1·84
	(28)	(28)	(28)	—
	24·80–28·65	13·37–16·65	1·248–1·538	1·61–1·98

* The figures represent the mean ± standard error of the mean, number of observations (in parentheses) and range

maintained on the stock diet. Whether or not this difference, if it occurred in the molars, would be of sufficient magnitude to have any effect on the incidence of dental caries is not known. If demineralization of tooth substance is a cause of caries (Miller, 1890), increased mineralization would not be any bar to the progress of the disease, in fact the better the calcification of the tooth the more likely would it suffer acid decalcification. Sobel & Hanok (1948) point out that comparatively small changes in the composition may affect tooth solubility, e.g. an increased content of carbonate at the expense of phosphate will result in an increased solubility in acid. It may well be that small changes in the composition of teeth are one of the many factors in the aetiology of dental caries.

The high-sugar diet affected both the enamel and the dentine, and its influence seems to have been twofold; first, the absolute amounts of calcium and phosphorus were raised, and secondly the calcium/phosphorus ratios of both tissues were lowered. This may mean that at the site of mineralization relatively less calcium was precipitated than phosphate, and therefore the composition of the fluid from which precipitation occurred had been modified.

In both groups of incisors the calcium/phosphorus ratios of the enamel were distinctly greater than those for the corresponding dentine. This finding agrees with that of Sobel & Hanok (1948). It has been a widespread belief that the mineral phases of enamel, dentine and bone are identical, but it would appear that for the rat this view must be modified, since the mineral phase of enamel has relatively less phosphate and more calcium than that of dentine. If we assume an apatite formula $3Ca_3(PO_4)_2$. CaX, in enamel X must consist mainly of radicals other than phosphate, whereas in dentine the proportion as phosphate must be considerable. Alternatively, if the basic apatite structure is the same in both tissues, the dentine must contain more adsorbed phosphate. In the rat the stoicheiometric calcium/phosphorus ratio is (except in one or two cases) greater than 1·5 for the enamel and less than 1·5 for the dentine.

Stoicheiometric calcium/phosphorus ratios are used throughout this paper, because they give a better understanding of chemical composition than a weight ratio. A stoicheiometric ratio of 1·5 is equivalent to a weight ratio of 1·94.

That there is some connexion between carbohydrate metabolism and mineralization is becoming increasingly evident. It has been known for some time that the developing tooth contains glycogen. Creighton (1896) described its occurrence in the rodent incisor, and a recent study by Bevelander & Johnson (1946) described its location in the tooth bud. Glycogen disappears when mineralization begins. Engel (1948) reported the presence of glycoprotein granules in the cytoplasm of the odontoblasts and ameloblasts in the stages prior to the formation of dentine and enamel. Glock (1940) suggests that the phosphorylation processes involved in the breakdown of glycogen are the first source of phosphate esters which set off the mineralization process; later when the glycogen has disappeared calcification continues by the action of the alkaline phosphatase on the phosphate esters of the blood. Boyle & Wesson (1943) made the interesting observation that the teeth formed on low-calcium diets in the presence of vitamin D were better when the diet was rich in carbohydrate than when it was rich in protein.

Our results show that a diet containing 67% sucrose leads to the formation of incisor teeth having a higher calcium and phosphorus content than those of animals receiving a stock diet.

The hypothesis is therefore advanced that a diet which is adequate in all known respects, and in addition is extremely rich in carbohydrate, may stimulate the mineralization process by maintaining the glycogen in the developing tooth at a maximum level. This hypothesis implies that local factors are more important in mineralization than humoral ones, which is probably true since, as Sobel & Hanok (1948) have observed, if only humoral factors were involved there should be no difference in the calcium/phosphorus ratios of enamel and dentine in the same animal. The present investigation has been concerned only with calcium and phosphorus since they are the two major inorganic components; variation in them will of course be accompanied by variation in certain other components, notably magnesium and carbonate. This will require further study. The analytical results for the enamel and dentine in both groups show considerable individual variation, and it is evident that the composition of the tooth is not rigidly fixed. Any study of tooth composition must receive statistical treatment.

The next stage in the investigation must be to study the effect of reducing the carbohydrate intake by replacing some of the sucrose in the purified diet with protein or fat and noting any significant change in the calcium and phosphorus content.

SUMMARY

1. The enamel and dentine of the incisors of rats maintained on a diet containing 67% sucrose had significantly higher amounts of calcium and phosphorus than the same tissues of animals maintained on a stock diet of Lever cubes.

2. The calcium/phosphorus ratios on the high-sugar diet were significantly lower than on the stock diet.

3. It is confirmed that in the rat the calcium/phosphorus ratio for the enamel is higher than that for the dentine.

4. The hypothesis is advanced that a diet high in sucrose stimulates the processes of mineralization by maintaining a maximum level of glycogen in the developing tooth.

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Chemistry of Tissues

4. METHYLATION STUDIES ON HYALURONIC ACID

By M. A. G. KAYE AND M. STACEY
Chemistry Department, The University of Birmingham

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Despite the many investigations on hyaluronic acid there is no precise knowledge of the structural units of which the complex polysaccharide is constituted. Accordingly, our investigation was undertaken in order to apply chromatographic technique, methylation studies, etc. to gain knowledge of hyaluronic acid structure.

Hyaluronic acid is extracted from human umbilical cords with water. Acidification of the extract yields a precipitate or mucin clot containing the hyaluronic acid in conjunction with protein. Most of the various procedures which have been employed for the isolation of hyaluronic acid have differed in the method of removal of this protein.

In early methods of isolation (Meyer & Palmer, 1934) both the cords and the mucin clot obtained therefrom were subjected to rather drastic acid treatment so that products of low viscosity and with low nitrogen and ash contents were usually obtained. Later (Meyer & Palmer, 1936), it was found that the Sevag chloroform-amyl alcohol method for protein denaturation (Sevag, 1934) could be applied to the dissolved mucin clot from which a hyaluronic acid product could then be precipitated from aqueous solution by acetic acid. Further treatment with zinc acetate and alkali effectively removed the protein residue. However, this treatment also reduced the viscosity of the product and it was found difficult to remove the zinc completely. It was necessary also to use dilute solutions owing to the danger of precipitating zinc hyaluronate.

Two general methods have been used to redissolve this clot: (*a*) 10% calcium chloride at pH 8, and (*b*) enzymic digestion at a more alkaline pH. Use of method (*a*) gave a product of slightly higher viscosity but in lower yield (Meyer & Chaffee, 1940*a*). McClean & Hale (1941) removed the protein residue by tryptic digestion followed by precipitation of the trypsin with trichloroacetic acid. Blix & Snellman (1945) extracted dissected cords with water in an atmosphere of nitrogen six or eight times for 12 hr. each. They found it necessary to apply the Sevag process many times before the last traces of protein could be removed and a product of high viscosity obtained.

Most of the procedures aimed at obtaining, on a small scale, a product of the highest possible viscosity. Since our work was completed (1947),

Hadidian & Pirie (1948) have reported yet a further modification in which they claim to have separated several fractions of hyaluronic acid having varying viscosities. Meyer (1948) has also described a method for preparing highly viscous sodium hyaluronate.

For the present chemical investigation a highly viscous preparation was not necessary, but a comparatively large quantity of material was required, and methods have been developed which are easily applicable on a large scale for the preparation of material for methylation.

EXPERIMENTAL

Preparation of hyaluronic acid and umbilical cord mucin. Human umbilical cords, preserved in 1% aqueous phenol solution, were washed thoroughly with cold water to remove blood clots, minced coarsely and extracted four or five times with hot water at 65° (total 5 l./30 cords) and filtered through cheese cloth. This solution was cleared by centrifuging and precipitated with glacial acetic acid (final concn. 0·2%) stirring vigorously. The fibrous precipitate was collected on a glass rod or by centrifuging and was dried over P_2O_5 in a vacuum. It contained N 14·8, ash 0%, and is termed the 'clot'. It was reprecipitated from an alkaline solution (pH 8·5) several times, the N content remaining constant at 15·0%; ash 1·1%. This preparation is regarded as umbilical cord mucin, a protein-polysaccharide complex (20 g. from 24 g. of clot). When dry it was very hard and brittle.

Hyaluronic acid. Method A. The mucin preparation (30 g.) was dissolved with stirring in a slight excess of aqueous K_2CO_3 (1 l., pH 8·5). $CHCl_3$ (1·25 vol.), containing 5% of *n*-butanol, was added and the mixture shaken vigorously for 4 hr. or longer (cf. Sevag, 1934). The two phases were separated in the centrifuge (2500 rev./min.). The aqueous layer was diluted to 2 l. with water and solid matter removed in the Sharples centrifuge (24,000 rev./min.). The supernatant liquid was poured, with stirring, in a fine stream into dry ethanol (2–3 vol.) and the white, fibrous precipitate was collected on a rod and dried in a vacuum over P_2O_5. This product (14 g.) contained N 10·8, ash, 3·3%.

One half of this material (7 g.) in water (800 ml., pH 8·5) was denatured with $CHCl_3$ repeatedly until no more protein was coagulated and the solution no longer gave a biuret test. After working up as described, and drying, a pure white, fibrous product, potassium hyaluronate, was obtained (2·3 g.) which gave viscous, water-clear, aqueous solutions: N (Kjeldahl) 3·2, acetyl (Elek & Harte, 1936) 12·3, uronic acid (Burkhart, Baur & Link, 1934), 44·9%. The hexosamine estimation by the method of Elson & Morgan (1933) was not satisfactory, high results frequently being obtained, namely, 52·9%; other values: 35·4, 35·0%. No *N*-methyl or *O*-methyl groups could be detected; $[\alpha]_D^{18°} - 68°$ in water (*c*, 0·22). The viscosity was determined in 0·05 M-NaCl and 0·05 M-phosphate buffer at pH 7·0. At 0·15 g./l. the relative viscosity (see Hadidian & Pirie, 1948) was 1·7; at 1 g./l. it was increased to 1·8. This value is low compared with some preparations (cf. Hadidian & Pirie, 1948), but it is the usual value for hyaluronate obtained from cords which have been extracted with hot water rather than with cold (Kaye, 1949). The electrophoretic pattern with one main peak, shown in Fig. 1, was exhibited in 0·05 M-Na_2CO_3 solution (total ionic strength increased to 0·2 with KCl), at pH 11·36. (The small peak on the left of Fig. 1 is due to the building up of a salt boundary.) A current of 10 m.a. was applied for 180 min. The rate of migration was slow, suggesting a molecule of low net charge. All analytical results are expressed on a dry, free acid basis. The sample contained ash 20·0% (K, 11·3%); moisture (air-dry sample), 10·5% (cf. Kaye, 1948). It contained no glycogen (cf. Meyer & Palmer, 1936) or phosphate, and was non-reducing.

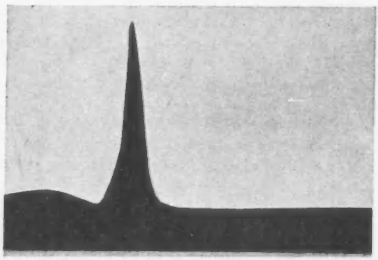

Fig. 1. Electrophoretic behaviour of the potassium hyaluronate preparation in 0·5% solution at pH 11·36 (0·05 M-Na_2CO_3, with KCl added to raise the ionic strength to 0·2) showing a symmetrical and homogeneous boundary after 9,420 sec. (Migration is from left to right.) The small peak is due to a salt anomaly.

This material (0·5 g.) was dissolved in water (200 ml.) and passed through a column of 'Zeo-Karb 215' to remove K ions. The rate of flow through the column was very slow, and if the original solution was too concentrated gel formation was found to prevent flow completely. The free acid could not be precipitated by pouring into ethanol, but the freeze-drying technique was found expedient and yielded hyaluronic acid as a tough, fibrous, spongy material (0·3 g.). Ash, 1·4; N, 3·6%. $[\alpha]_D^{17°} - 67°$ in water (*c*, 0·3). The relative viscosity (see above) at 0·15 g./l. was 1·5.

Hyaluronic acid. Method B. The umbilical cord mucin preparation (22 g.) was dissolved in dilute aqueous KOH (700 ml., pH 8·5). The solution was boiled for a few minutes with $BaCO_3$, cooled and centrifuged. The clear supernatant liquid was poured into ethanol (4 vol.), the precipitate separated in the centrifuge and dried in a vacuum over P_2O_5. It was a yellowish white powder (18 g.); N, 7·9%, $[\alpha]_D - 56°$ in water (*c*, 0·3). This material was heated again in water (600 ml.) with $BaCO_3$ (25 g.) for a further period of 6 hr. at about 80°. The insoluble matter was separated in the centrifuge and the supernatant liquid was poured into ethanol (4 vol.). The white, fibrous precipitate was collected on a rod, and dried in a vacuum over P_2O_5. It was re-extracted with water, precipitated and dried again. This preparation (10·5 g.) showed $[\alpha]_D - 30·6°$ in water (*c*, 0·72); N, 3·7; ash, 19·1%. The relative viscosity (see above) was 1·3 at both 0·15 and 1·0 g./l. This preparation was regarded as depolymerized hyaluronic acid and was a typical product.

Hydrolysis of potassium hyaluronate by acid. A portion of the potassium hyaluronate preparation (1·0 g.) in 0·1 N aqueous HCl (60 ml.) was heated in a water bath for several hours. There was no change in rotation during the first 8 hr. and then only a slow change after a further 8 hr. $[\alpha]_D \rightarrow -12°$. At this stage the acid concentration was increased to 1·0 N. Reaction then proceeded more rapidly: $[\alpha]_D - 12° \rightarrow +24°$

(max.) in 50 hr. The hydrolysate gave positive tests for carbohydrate, reducing sugar, amino sugar (Elson & Morgan, 1933) and uronic acid (naphthoresorcinol). White crystals of an amino sugar were isolated from the hydrolysate. These were separated and recrystallized from methanol-acetone mixture (0·25 g.) (cf. Meyer & Palmer, 1936). (Found: $[\alpha]_D^{21°}+67°$ (constant after 18 hr.) in water; *c*, 1·82.) Glucosamine hydrochloride requires $[\alpha]_D^{20°}+72·5°$, equilibrium value in water. It was identified by formation of *N*-acetyl glucosamine (decomp. about 190°; $[\alpha]_D-40·9°$ in water) and also by an X-ray powder photograph (cf. Cox & Jeffrey, 1939).

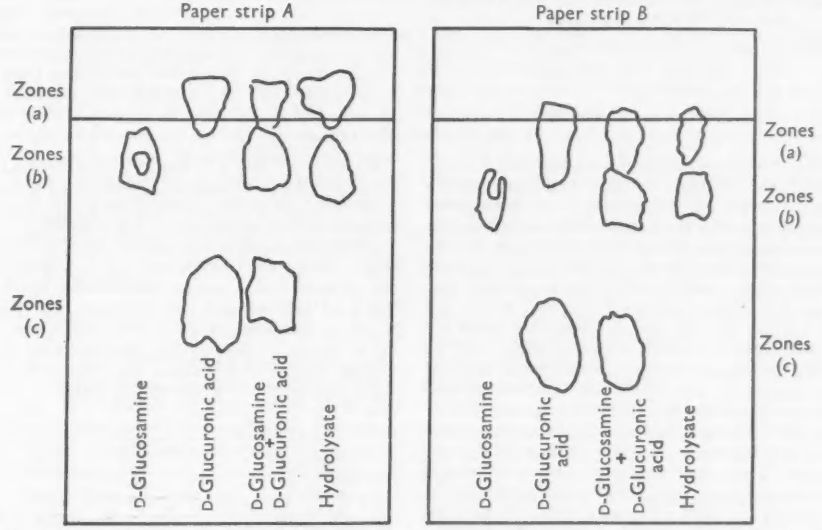

Fig. 2. A diagrammatic reproduction of the paper-strip chromatograms comparing a hydrolysate of potassium hyaluronate with authentic substances. Both strips were irrigated for 12 hr. (*A* with *n*-butanol-ethanol, *B* with *n*-butanol-acetic acid) and developed with ammoniacal $AgNO_3$.

Chromatography of the hydrolysate. The conditions were determined which gave the maximum hydrolysis of potassium hyaluronate with the minimum decarboxylation of the uronic acid. Accordingly, potassium hyaluronate (0·14 g.) in N-H_2SO_4 (10 ml.) was maintained just below boiling point for 8 hr. It was exactly neutralized with N-NaOH (using a volume predetermined by a control titration) and concentrated under reduced pressure to small volume (1 ml. approx.). The hydrolysate was examined by the filter paper strip method as used by Partridge (1946) for reducing sugars. Two papers containing a drop (0·01 ml.) of a 2% aqueous solution of each of the following were irrigated for 12 hr., one with *n*-butanol-ethanol and the other with *n*-butanol-acetic acid. Both were dried at 105°, developed with ammoniacal $AgNO_3$ and heated at 105° for 10 min.: (i) D-glucosamine, (ii) D-glucurone, (iii) D-glucosamine + D-glucurone, (iv) hydrolysate (see Fig. 2). On both strips the hydrolysate gave rise to a zone corresponding with that given by authentic D-glucosamine. Authentic D-glucurone gave rise to an upper zone (*a*) due to the free acid formed from the lactone and to a lower zone (*b*) due to the lactone itself (cf. Partridge, 1948). The hydrolysate gave rise to an upper zone only, corresponding with that from D-glucuronic acid. Table 1 indicates the similarity of the R_F values of the hydrolysate zones to those of D-glucuronic acid and D-glucosamine respectively.

By irrigating a strip containing two separate drops of hydrolysate, cutting the strip longitudinally so that each drop then occupied a separate narrow strip, and by de-

Table 1. *Comparison of R_F values of the zones on the paper chromatograms*

	Paper A (solvent boundary 20·1 cm.)				Paper B (solvent boundary 26·4 cm.)			
	Zones (*a*)		Zones (*b*)		Zones (*a*)		Zones (*b*)	
	Distance (cm.)	$R_F \times 10$	Distance (cm.)	$R_F \times 10$	Distance (cm.)	$R_F \times 10$	Distance (cm.)	$R_F \times 10$
D-Glucosamine	—	—	1·2	0·60	—	—	2·5	0·95
D-Glucurone	−0·5	−0·25	—	—	0·7	0·27	—	—
D-Glucosamine + D-Glucurone	−0·5	−0·25	1·2	0·60	0·6	0·23	2·4	0·91
Hydrolysate	−0·6	−0·30	1·5	0·75	0·5	0·19	2·4	0·91

veloping only one of these narrow strips, the position of the zones on the undeveloped strip was determined by comparison with the developed strip. These undeveloped zones were cut out and eluted with water. In this way the upper zone (*a*) was found to give a positive naphthoresorcinol test confirming uronic acid and the lower zone (*b*) gave a positive Elson-Morgan test confirming hexosamine.

Methylation of hyaluronic acid. The depolymerized hyaluronic acid preparation (10·2 g.) was dissolved in water (70 ml.), mixed with CCl_4 (300 ml.) and 35% aqueous NaOH (21 ml.) and stirred vigorously at room temperature. Methylation was effected by addition of dimethyl sulphate (80 ml.) and 35% NaOH (149 ml.) at 10 min. intervals over a period of 1 hr., vigorous stirring being maintained throughout. The temperature was raised to 35° and dimethyl sulphate (75 ml.) and 35% NaOH (160 ml.) were added at 10 min. intervals over a period of 2 hr., stirring continuously for a further 5 hr. Excess dimethyl sulphate was then destroyed by boiling and the CCl_4 distilled off. Stirring was continued while the mixture was cooled to 0° and neutralized with 25% H_2SO_4. The Na_2SO_4 was separated by crystallization and precipitation with ethanol and the supernatant liquid was concentrated to a thin brown syrup. This product was stirred at 50° with CCl_4 (260 ml.) and 35% NaOH (11 ml.). Methylation proceeded with addition of dimethyl sulphate (40 ml.) and 35% NaOH (74 ml.) at 10 min. intervals over a period of 1 hr. Stirring was continued at 50° for 6 hr. The methylation process was repeated and the mixture cooled and neutralized and the product isolated as described. Six further methylations at 50° were effected similarly in pairs.

To protect free amino groups, the methylated product was acetylated with acetic anhydride (12 ml.) in dry methanol (120 ml.) at room temperature for 48 hr. The solvents were removed at 40° and the product 'dried' in a vacuum over P_2O_5 and KOH for several days. This material, in methyl iodide (30 ml.), containing just sufficient dry acetone (5 ml.) to effect solution, reacted vigorously in the cold upon the addition of Ag_2O (1 g.), due to the rapid methylation of free carboxyl groups. After refluxing for 8 hr. the Ag residues were removed in the centrifuge and the supernatant liquid was concentrated. (4·18 g. OMe, 25·6; N, 2·6%. Mol. wt. 720 approx., in camphor.) This molecular weight is essentially a minimum value owing to the poor solubility of the product in camphor.

This product, in dry methanol (50 ml.), was acetylated again with acetic anhydride (5 ml.) in the presence of silver acetate (1 g.) for 48 hr. at room temperature. The Ag salts were removed in the centrifuge and the supernatant liquid was concentrated and dried over KOH in a vacuum. This product was methylated in methyl iodide (20 ml.) in the presence of Ag_2O (2 g.) added in two portions during 8 hr. The product was isolated in the usual manner and the methylation repeated (OMe, 32·9%.) This figure was not increased after further methylation. Yield, 2·75 g.

Fractionation and methanolysis of methylated hyaluronic acid

The methylated polysaccharide in 1 vol. of $CHCl_3$ (10 ml.) was fractionally precipitated by light petroleum. Four fractions were obtained (see Table 2).

Fraction *F*4 (1·27 g.) was refluxed with 3% methanolic HCl (100 ml.) (see Fig. 3, curve *b*). After 34 hr. the solution was neutralized with Ag_2CO_3 and the filtered solution concentrated to a thin syrup, 1·10 g. (OMe, 37·3%.)

Table 2. *Fractional precipitation of methylated depolymerized hyaluronic acid from chloroform solution by light petroleum* (60–80°)

Fraction	Precipitant volume	Wt. (g.)	Ash (%)	OMe (%)	$[\alpha]_D$
*F*1	1	0·12	55·4	24·1*	+12·4°* in $CHCl_3$ (*c*, 0·57)
*F*2	6 (+5)	0·59	0·0	29·0	+17·9° in $CHCl_3$ (*c*, 1·06)
*F*3	21 (+15)	0·29	0·0	29·0	+91·9° in $CHCl_3$ (*c*, 1·03)
*F*4	∞	1·29	0·0	33·7	+ 8·9° in methanol (*c*, 1·29)

* Calculated on ash-free basis.

Fractions *F*1 and *F*2 were combined (0·61 g.) and refluxed with 3% methanolic HCl (60 ml.) (see Fig. 3, curve *a*). After 30 hr. the product was isolated in the usual way and concentrated to a syrup, 0·47 g. (OMe, 33·3%.)

Fractionation of the methanolysates. Fraction *F*4 methanolysate (1·10 g.) was distilled in a high vacuum and yielded two fractions: (*a*) a mobile, ether-soluble fraction, *F*4*a*, 0·41 g. at 120–140° and 0·01–0·007 mm. Hg (OMe, 46·2; N, 0·2%; n_D 1·4560; $[\alpha]_D^{17.5°}$ +3·8° in $CHCl_3$ (*c*, 1·31)); and (*b*) a viscous, ether-insoluble fraction, *F*4*b*, 0·26 g. at 210–240° and 0·005–0·004 mm. Hg (OMe, 27·3; N, 0·9%; n_D 1·4835; $[\alpha]_D^{17.5°}$ +7·4° in $CHCl_3$ (*c*, 0·94)).

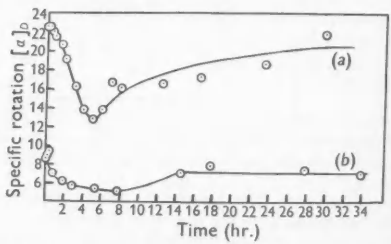

Fig. 3. Hydrolysis of the methylated depolymerized hyaluronic acid. (*a*) Fraction *F*2; (*b*) fraction *F*4.

Fraction *F*4*a* was methylated with methyl iodide and Ag_2O (×6) to OMe 50·2% (constant). The product, in acetone (5 ml.), was passed through a column of activated Al_2O_3 and the eluted solution collected in 2 ml. portions which were grouped into fractions according to the optical rotation. The first fraction was concentrated and re-esterified with diazomethane in ether to ensure that no carboxyl groups, liberated by the catalytic action of the Al_2O_3, remained free (cf. Strain, 1942). The ether was removed and the amide formed from the ester in methanolic NH_3. The solvents were removed at 0° and the product (20 mg.) dissolved in acetone from which a small crop of crystals was obtained. $[\alpha]_D^{20°}$ +136° in acetone (*c*, 0·5); m.p. and mixed m.p. with authentic 2:3:4-trimethyl-α-methyl D-glucopyruronoside acid amide 179°.

Fraction *F*1–*F*2 methanolysate (0·47 g.) was acetylated and remethylated several times in the usual manner without increasing the methoxyl content (33 %). The product was distilled in a high vacuum yielding two fractions, *F*2*A* (81 mg.) and *F*2*b* (23 mg.), which resembled the fractions *F*4*a* and *F*4*b* respectively in solubility and viscosity.

Fraction *F*2*a* was methylated twice with methyl iodide and Ag_2O and the amide formed in methanolic NH_3. The solution was concentrated to a syrup at 0° and dissolved in dry acetone (1 ml.) when a small quantity of crystalline material separated, resembling in crystalline form the fraction *F*4*a* and the authentic 2:3:4-trimethyl-α-methyl D-glucopyruronoside acid amide: m.p. and mixed m.p. with the authentic material 174–175°.

The ether-insoluble fractions. Fractions *F*2*b* and *F*4*b* were combined, acetylated and methylated to constant methoxyl content (×4) with methyl iodide and Ag_2O. The product (0·29 g.) contained OMe, 37·4; N, 1·8 %. This material would not crystallize and the low N content suggested that the main part of the glucosamine residue had remained undistilled; therefore the two still residues were combined and extracted with $CHCl_3$. The extract was a dark, viscous liquid (0·18 g.). OMe 19·1; N, 6·1 %. Mol.wt. 327 ± 10. (Calculated for a dimethyl *N*-acetyl glucosamine: OMe, 25; N, 5·6 %; mol.wt. 283.) The methoxyl content could not be increased by further methylation or by methanolysis. It was non-reducing to Fehling's solution.

RESULTS AND DISCUSSION

It is interesting to compare the physical nature of the umbilical cord mucin and potassium hyaluronate preparations. Both form a stringy meshwork in the wet state when precipitated from solution, but when dried the hyaluronate assumes a flexible, fibrous nature whilst the mucin shrinks to a very hard and compact mass which is very tough and brittle and which can be ground to a granular powder. The depolymerized hyaluronate is precipitated in flocks, not fibres, and dries to a fluffy powder of very open texture. It is well known that considerable cross-linking between long chains leads to a brittleness of the product. It is suggested that the physical nature of the mucin is due to considerable cross-linking of the hyaluronic acid and protein to form a fairly rigid three-dimensional structure. On this account it might be thought feasible that such a complex will have a greater viscosity than any one component separated from it (cf. Meyer & Chaffee, 1940*b*): Hadidian & Pirie (1948) found that removal of protein lowers the viscosity. Recently, Ogston & Stanier (1950) reported the isolation of a hyaluronic acid-protein complex from synovial fluid by an ultrafiltration procedure, having the same viscosity as the fluid, but removal of protein was found to cause a lowering of the viscosity.

Although the viscosity of the potassium hyaluronate was low, the relative viscosity increased slightly with increase in concentration, which phenomenon is characteristic of the more highly polymerized samples (cf. McClean & Hale, 1941; Blix & Snellman, 1945; Hadidian & Pirie, 1948). On the other hand, the relative viscosity of the depolymerized preparation bore a linear relationship to the concentration.

The hyaluronic acid obtained from the potassium salt was submitted to Prof. W. T. Astbury for X-ray examination, but the X-ray photographs were unsatisfactory and the substance showed no crystalline form. This is in accordance with the conclusions of Blix & Snellman (1945) that hyaluronic acid has no very highly ordered structure. However, the relative viscosity was found to have been decreased by contact with the 'Zeo-Karb 215' and further samples are being prepared suitable for X-ray examination.

To the authors' knowledge, the paper chromatographic results provide the first direct evidence other than that of a colour test for the presence of uronic acid in an acid hydrolysate of hyaluronic acid. Meyer has isolated and characterized its lead salt from an enzymic hydrolysate (Meyer, Smyth & Dawson, 1939) and its presence in hyaluronic acid has been inferred also from the isolation of a saccharic acid derivative as an oxidation product (Meyer & Palmer, 1936; Kendall, Heidelberger & Dawson, 1937). It seems that many uronic acids are very slow-moving when irrigated on paper. Also, their R_F values are very close, so that the chromatographic results cannot be regarded as providing conclusive evidence for the presence of glucuronic acid specifically.

A protein-free product containing 3·7 % nitrogen could be obtained from the mucin by treatment with barium carbonate. This method of isolation was selected as a milder modification of a more usual, but drastic, procedure of separating polysaccharides from protein matter using sodium hydroxide, since the latter is known to cause severe degradation of hyaluronic acid (cf. Kaye, 1948). The product had a lower specific rotation than the potassium salt obtained by the Sevag procedure and its aqueous solutions were less viscous, suggesting that a certain amount of depolymerization had occurred, which was not apparent in preparations obtained by less drastic methods. However, as the object of the methylation technique for investigating polysaccharides was the recognition of the position of glycoside linkages present, and as these are normally stable to alkaline conditions and not involved in depolymerization brought about by alkali, this product, easily obtained in large quantities, was regarded as suitable for a first investigation.

The Freudenberg methylation procedure (Freudenberg & Boppel, 1938) in liquid ammonia failed to give a satisfactory product and was abandoned. The methylated product obtained in 30 % yield by the Haworth (1915) technique was more satisfactory. The methoxyl content (33 %) corresponds to 4·2 groups/glucuronic acid-*N*-acetyl glucosamine disaccharide anhydro unit of an endless chain. Theory

requires five such groups and 2·7 % nitrogen. Although further methylation could not be effected by the methods used, the following reasoning regarding the fractionation of the product by light petroleum seems to show that it was probably not fully methylated. Fractions *F* 1, *F* 2 and *F* 4 are almost certainly derived from the same molecular species differing slightly in extent of methylation, the least methylated being precipitated first. While fraction *F* 3 is apparently different from the others, probably more degraded, it constitutes only 10 % of the product. Thus, assuming fractions *F* 1, *F* 2, and *F* 4 to be of similar type, it is apparent that fractions *F* 1 and *F* 2 cannot be fully methylated.

Analysis results of the undistilled residue of methanolysed fraction *F* 4 simulate those for a dimethylated glucosamine derivative with possibly a small percentage of dimer. The smaller methoxyl content and higher nitrogen content of fraction *F* 4*b* than *F* 4*a* also suggests that the former contains a greater percentage of a partially methylated glucosamine. Furthermore, these glucosamine units could not be more fully methylated even after further methanolysis and acetylation of the amino nitrogen. It may be noted that glucosamine in a straight chain has two exposed hydroxyl groups available for methylation; methanolysis will produce a third one. Thus, the formation of dimethyl glucosamine derivatives indicates one of three possibilities: (*a*) branching of the chain at the glucosamine unit, (*b*) rupture of the glycoside link without the introduction of a methyl group, although how this will occur in the absence of water is not quite clear, (*c*) only partial methylation of the original polysaccharide itself, in which case, characterization of the partially methylated glucosamine derivatives cannot lead to conclusions regarding the structure of the polysaccharide molecule.

The authors are of the opinion that, for certain reasons, methylation of the hyaluronic acid molecule was incomplete; and, probably for the same reasons, complete methylation of the glucosamine moiety could not be effected. These reasons may be connected in some way with the amino group and its acetylated form. The state of combination of the acetyl group in most naturally occurring polysaccharides containing glucosamine is not yet fully understood. For example, it has been reported (Wolfrom, Weisblat, Karabinos, McNeely & McLean, 1943) that the amino group of heparin is neither free nor acetylated and a recent publication (Jorpes, Boström & Mutt, 1950) suggests an —NH—SO_2OH grouping. Hahn (1946) claims to have isolated *N*-acetyl glucosamine from hyaluronic acid after an enzymic hydrolysis, but it has never been isolated from an acid hydrolysate of the polysaccharide. Furthermore, although it has never been found possible to methylate glucosamine itself owing to the electron-donating properties of the amino group, *N*-acetyl glucosamine can be methylated (see Moggridge & Neuberger, 1938); however, attempts to methylate chitin, the supposed poly-*N*-acetyl glucosamine, have not met with any success (Stacey, 1944). It seems, then, that the position and effects of the acetyl group, or the grouping which will give acetic acid on hydrolysis, in this type of molecule require investigation.

Regarding fraction *F* 4*a*, it may safely be assumed that the nitrogen belongs to a glucosamine unit. This being so, presumably it will be more completely methylated than the dimethyl derivative which would not distil under these conditions. If it were a trimethyl *N*-acetyl glucosamine it would constitute only 3·8 % of the fraction, and allowing for this, the remaining glucuronic acid derivative would have a 47 % methoxyl content. This is the value required for a tetramethyl glucuronic acid derivative. This must originally have occupied a central position in a chain, since an end unit of this type (necessarily linked through carbon 1) would yield a fully methylated derivative which would contain 56 % methoxyl. (Upon methylating fraction *F* 4*a*, the methoxyl content was raised to 50·2 % only. This may well explain why only a small quantity of the fully methylated acid amide was obtained.)

The methoxyl content of the combined fractions *F* 2*b* and *F* 4*b* indicates that the glucuronic acid was probably the trimethyl but certainly not the fully methylated derivatives. Thus, 2·75 g. of the methylated product yielded, in four fractions, 2·3 g., of which 2 g. contained non-end-group glucuronic acid. The methylated product was obtained in 30 % yield; therefore, it can be concluded that at least one-third of the glucuronic acid in hyaluronic acid occurs in a 'central' position in the chain and, unless the losses during methylation included selective rupture of end-group glucuronic acid units, it might be suggested as an hypothesis, that only a very few, if any, of the glucuronic acid groups occupy an end position (cf. Kaye & Stacey, 1950).

The type of linkage that combines the protein and carbohydrate molecules in umbilical cord mucin, whether the latter be naturally occurring or artificial, is unknown, it having been presumed to be merely a salt type. Recently, Ogston & Stanier (1950) have reported the existence of a firm linkage between protein and hyaluronic acid in synovial fluid. It was considered, therefore, that the methylation of the cord mucin might provide certain indications of the presence or absence of covalent linkages between protein and polysaccharide. A methylated product was obtained from the mucin containing 18 % methoxyl, 6·6 % nitrogen and 5 % acetyl. Thus, only about one-half of the product could be accounted for by the hyaluronate moiety, the remainder containing about 10 % nitrogen, being

presumably of protein origin. After methanolysis a small unidentified fraction containing 12·5% nitrogen was obtained, in fact no fraction was found to contain less than 6·5% nitrogen. Further treatment failed to produce any nitrogen-free fractions.

Stacey & Woolley (1940) were able to remove all of the protein from ovomucoid during methylation. This was not so in the present case, however, and in view of the recent publications of Gottschalk & Partridge (1950*a*, *b*) reporting the interaction of simple sugars and proteins, we suggest, by way of explanation, that some irreversible reaction may have occurred between the carbohydrate and protein moieties of the methylated product at some stage after the methylation.

Until further information is available concerning the possible interaction between protein and carbohydrate molecules under varying conditions, the mucin must be regarded as an unsuitable starting material for chemical investigations.

SUMMARY

1. Potassium hyaluronate has been prepared from human umbilical cords and the free acid obtained therefrom using 'Zeo-Karb 215' to remove the potassium ions. Both were obtained in fibrous form.

2. The hyaluronic acid preparation has been submitted to X-ray examination, but it gave no satisfactor X-ray photographs and showed no very highly ordered structure.

3. From an aqueous acid hydrolysate of potassium hyaluronate glucosamine hydrochloride was crystallized and identified by an X-ray powder photograph. The presence of glucosamine and a uronic acid in the hydrolysate was confirmed by application of the paper-strip chromatographic technique.

4. A partially depolymerized hyaluronate preparation was obtained and methylated with dimethyl sulphate followed by methyl iodide, to yield a product containing 33% methoxyl. From this, a uronic acid derivative was isolated and identified, after further methylation, as 2:3:4-trimethyl α-methyl-D-glucopyruronoside acid amide. At least one-third of the uronic acid residue in the hyaluronic acid molecule is present in a chain, linked glycosidically through its reducing group and one other hydroxyl group and is not present as an end group. No amino sugar derivatives could be identified from the methylated product, the significance of which is discussed.

5. Methylation with sodium and methyl iodide in liquid ammonia (Freudenberg) was unsatisfactory and the method was considered unsuitable for hyaluronic acid.

We wish to thank Mr D. Patrick for carrying out the electrophoresis examination. Our thanks are due also to the Medical Research Council and to Messrs Imperial Chemical Industries Ltd. for financial assistance.

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